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Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendments, claims 1-16 are pending in the application, with claims 7-10 withdrawn from consideration, and claims 1, 7 and 11 being the independent claims. Claim 1 has been amended to recite the terms "inhibits" and "inhibiting." Support can be found, for example, at page 9, lines 18-28. New claims 11-16 have been added. Support for new claims can be found in the originally filed claims 1-6 and at page 9, lines 18-28. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendments and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Supplemental Application Data Sheet

Submitted herewith is a Supplemental Application Data Sheet (ADS) under 37 CFR § 1.76(c), § 1.76(d)(3), § 1.76(d)(4), § 1.48(f)(1) and MPEP § 601.05. The Supplemental ADS supplements information in the original ADS, which was filed with the captioned application, with bibliographic information for the third inventor, Wolfgang Sommergruber, which was provided in the original declaration filed on June 20, 2002 along with the Reply to the Notice to File Missing Parts. Under 37 CFR § 1.76(d)(4) and MPEP § 601.05, Applicants hereby request the PTO to recapture the information from the Supplemental ADS.

Nucleotide Sequence and/or Amino Acid Sequence Disclosures

The Examiner states that the application fails to comply with the requirements of 37 CFR § 1.821 through § 1.825 and requires that an initial computer readable form (CRF) copy of the "Sequence Listing," and an initial paper copy or compact disc copy of the "Sequence Listing" be provided along with an amendment directing the entry of the Sequence Listing into the application.

Applicants previously filed a Substitute Sequence Listing on June 20, 2002, which contained minor typographical errors. Specifically, line 160 incorrectly indicated that "12" sequences were being submitted instead of "13." Further, in line 120, in the title, the words "that modulate" have been replaced with the word "modulating." A corrected paper and computer-readable copy of a Second Substitute Sequence Listing is filed with this Amendment. Further, in line nos. 140 and 141 of the Second Substitute Sequence Listing, the Application Number of the captioned application and the filing date, respectively, are provided. The content of the sequence listing information recorded in the computer readable form is identical to the paper copy form, and includes no new matter.

It is respectfully requested that objections under 37 CFR § 1.821 through § 1.825 be withdrawn.

Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 1-5 were rejected under 35 U.S.C. § 112, first paragraph, allegedly for lack of written description. Applicants respectfully traverse this rejection.

The standard for determining compliance with the written description requirement is that the applicant must "convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession *of the invention*. The invention is, for

purposes of the "written description" inquiry, *whatever is now claimed.*" *See*, *Vas-Cath Inc. v. Mahurkar,* 935 F.2d 1555, 1563-64, 19 USPQ 2d 1111, 1117 (Fed. Cir. 1991).

According to the Examiner, the specification does not provide a written description of any method for identifying compounds that modulate sister chromatid separation using any separase or fragment thereof of any amino acid sequence/structure and biological order and any substrate of any amino acid sequence/structure.

The captioned application claims priority to EP 01 101 252.3, filed January 19, 2001, and claims the benefit of U.S. Appl. No. 60/297,440, filed June 13, 2001. Applicants respectfully submit that the inventors had possession of the invention as of the priority date of January 19, 2001.

(1) Separase or Fragments thereof:

Applicants respectfully disagree with the Examiner as follows.

The specification discloses that separase is a protease. *See*, p. 1, line 23. Proteolytic activities of separase include the cleaving of a complex of chromosomal cohesion proteins referred to as the cohesion complex that holds sister chromatids together in the cell. *See*, p. 1, lines 23-25. Cleavage of the cohesion complex separates the sister chromatids from each other so that they can be pulled towards the opposite poles of the dividing cells by the spindle apparatus. *See*, p. 1, line 25 to p. 2, lines 1-3. Furthermore, separase has autocatalytic activity. *See*, p. 5, lines 15-17.

The claims are not directed to any separase or fragments thereof. The claims are directed to a screening method using *an active* separase or fragments thereof. *See*, p. 5, lines 21-28. Hence the separase fragment must have sufficient activity to work in the claimed method.

The Examiner stated that the specification only provides written description for human recombinant separase. To the contrary, the specification discloses yeast separase. *See*, p. 3, lines 1-4. Furthermore, the specification also cites documents that disclose separases from sources other than human and yeast. *See*, p. 3, lines 4-5 and 13-14, and references therein.

Uhlmann *et al.*, *Cell 103*:375-386, 378, Fig. 4 (October 27, 2000) (Exhibit A), discloses the following separases:

S. cerevisiae	Esp1 (AAB03897);
S. pombe	Cut1 (A35694);
C. elegans	AAA83576 and the WormPep-Entry CE22098;
E. nidulans	BimB (P33144);
D. melanogaster	conceptual translation of AC0114811;
H. sapiens	BAA11482; and
A. thaliana	CAA19812.

Uhlmann *et al. Nature 400*:37-42, 42 (July 1, 1999) (Exhibit B), discloses yeast separase Esp1 and cites documents that disclose separases from other species, for example:

Aspergillus nidulans	May, G.S. *et al.*, *J. Biol. Chem. 267*:15737-15743 (1992);
S. cerevisiae	McGrew *et al.*, *Mol. Biol. Cell. 3*:1443-1454 (1992); and
Fission yeast	Uzawa *et al.*, *Cell 62*:913-925 (1990).

The specification discloses several fragments of separase, for example:

recombinant separase fragment lacking the N-terminal, KIAA0165 (*see* p. 6, lines 11-14);

recombinant N-terminal separase fragment (*see* p. 6, lines 14-15);

N-terminal separase fragment, from amino acid (aa) 1 to 1506 (*see* p. 6, line 9 and Fig. 7);

N-terminal separase fragment, from aa 1 to 1533 (*see* p. 6, line 9 and Fig. 7);

C-terminal separase fragment, p60, from aa 1507 to 2120 (*see* p. 7, line 18 and Fig. 7); and

C-terminal separase fragment, p55, from aa 1536 to 2120 (*see* p. 7, line 18 and Fig. 7).

Further the specification, at p. 7, lines 9-10, cites Waizenegger *et al.*, *Cell 103*:399-410, (October 27, 2000) (Exhibit C), which discloses several separase fragments:

anaphase-specific cleavage products of separase (*see* Fig. 3B, p. 403, lines 12-14 of Fig. 3 description);

180 kDa C-terminally truncated form of separase (*see* p. 405, Fig. 6 (A and B), star symbol; p. 405, col. 1, line 1);

mitosis-specific C-terminal separase cleavage products (*see* Fig. 6 (A and B), open arrowheads);

C-terminal separase fragment, p60 (*see* p. 405, col. 2, 1st line of 2nd ¶; p. 405, col. 2, last line to p. 406, col. 1, lines 1-4); and

C-terminal separase fragment, p55 (*see* p. 405, col. 2, 1st line of 2nd ¶; p. 405, col. 2, last line to p. 406, col. 1, lines 1-4).

(2) Separase Substrates:

The Examiner stated that the specification does not provide written description for any substrate of any amino acid sequence/structure. Applicants respectfully disagree.

The claims are not directed to any substrate of any amino acid sequence/structure. The claims are directed to a screening method using separase substrates. Hence, the claims require a separase substrate that will work in screening compounds.

Further, the specification discloses several separase substrates and their derivatives:

SVEQGR;

DREIMR;

SFEILR;

EWELLR;

Bio-SVEQGR-amk;

Bio-SVEQGR-amk;

Bio-DREIMR-amk; and

DREIMR-amk.

See, p. 5, lines 19, 22 and 26; p. 12, lines 27-28 and p. 17-18.

Further, the specification cites documents that disclose other separase substrates, e.g., an 18 amino acid peptide MDDREIMREGSAFEDDDM, which contains the separase cleavage site, or a mutation or a fragment thereof. *See*, p. 5, line 11; WO 00/48627, p. 20, lines 28-31 (August 24, 2000) (Exhibit D).

Furthermore, a consensus sequence, SxExGRR that represents the site for the proteolytic cleavage of Scc1 by separase, and several potential sites that can be cleaved by separase were known:

SLEVGRR;

SVEQGRR;

SVERGRK;

SHEYGRK;

SIEAGR;

SIEVGR; and

EVEVGR.

See, Uhlmann *et al.*, *Nature 400*:37-42, 41, Fig. 7 (July 1, 1999) (Exhibit B).

In addition, Parisi *et al.*, *Mol. Cell. Biol. 19*:3515-3528, 3523, Fig. 5 (May 1999) (Exhibit E), discloses separase substrates from the following sources:

S. cerevisiae;

S.pombe;

C. elegans;

human; and

mouse.

Applicants respectfully submit that given the teaching in the captioned application one of ordinary skill in the art could have obtained separases and separase fragments from several sources other than human recombinant separase, and synthesized other separase substrates useful in the claimed screening method.

In view of the above, it is respectfully requested that the rejections under 35 U.S.C. § 112, first paragraph, be withdrawn.

Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 1-6 were rejected under 35 U.S.C. § 112, second paragraph allegedly for being indefinite.

The Examiner stated that the phrase "modulating sister chromatid separation" renders claim 1 (and claims 2-6 depending therefrom) "vague and indefinite because the meaning of the phrase is not known or recited."

Solely to expedite prosecution, claim 1 has been amended to recite "inhibits" and "inhibiting."

It is respectfully requested that the rejections under 35 U.S.C. § 112, second paragraph, be withdrawn.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Cleavage of Cohesin by the CD Clan Protease Separin Triggers Anaphase in Yeast

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Summary

In eukaryotic cells, replicated DNA strands remain physically connected until their segregation to opposite poles of the cell during anaphase. This "sister chromatid cohesion" is essential for the alignment of chromosomes on the mitotic spindle during metaphase. Cohesion depends on the multisubunit cohesin complex, which possibly forms the physical bridges connecting sisters. Proteolytic cleavage of cohesin's Scc1 subunit at the metaphase to anaphase transition is essential for sister chromatid separation and depends on a conserved protein called separin. We show here that separin is a cysteine protease related to caspases that alone can cleave Scc1 in vitro. Cleavage of Scc1 in metaphase arrested cells is sufficient to trigger the separation of sister chromatids and their segregation to opposite cell poles.

Introduction

Accurate chromosome segregation is crucial for the proliferation of somatic cells and for the production of haploid gametes during meiotic divisions. Mistakes during the chromosome segregation process give rise to cells with too many or too few chromosomes. This state, known as aneuploidy, is associated with most malignant tumor cells and is the leading cause of mental retardation (Down's syndrome) and spontaneous fetal abortion (Griffin, 1996; Lengauer et al., 1997).

The segregation of chromosomes to opposite poles of the cell requires their prior alignment on the mitotic spindle. During this process, sister chromatids condense, partially but not completely separate from each other, and attach to microtubules that extend to opposite poles of the cell. This culminates in a state called metaphase, during which sister chromatids come under tension from spindles attempting to pull them in opposite directions. Soon after all pairs of sister chromatids come under tension, chromosomes suddenly split and sister chromatids segregate to opposite halves of the cell; this period of mitosis is called anaphase (reviewed in Nasmyth et al., 2000). The metaphase to anaphase transition is one of the most dramatic and highly regulated steps in the process by which eukaryotic cells disseminate their genomes during cell proliferation. Its molecular basis has long remained elusive.

It has long been recognized that metaphase is a state of equilibrium (Mazia, 1961) during which sister chromatid splitting due to traction exerted by microtubules on kinetochores is counteracted by connections that hold sisters together. It is also clear that sister separation is an autonomous process that frequently still occurs even when poisons destroy the spindle (Mole-Bajer, 1958). These observations have led to the notion that chromosome segregation during anaphase might be triggered not by any change in the force exerted by microtubules, but rather by the sudden loss of cohesion holding sister chromatids together. Evaluation of this hypothesis, however, has had to await discovery of the apparatus that holds sisters together during metaphase.

Recent studies have identified a multisubunit complex, called cohesin, which is essential for holding sister chromatids together in organisms as diverse as yeast and *Xenopus* and is a good candidate for the substance that forms the bridge between sisters (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998; Toth et al., 1999). Cohesin functions from S phase until anaphase. It is both necessary for establishing sister chromatid cohesion during DNA replication (Uhlmann and Nasmyth, 1998) and for maintaining connections between sisters during metaphase (Ciosk et al., 2000).

In yeast, cohesin is tightly associated with chromosomes from DNA replication until the metaphase to anaphase transition (Michaelis et al., 1997; Toth et al., 1999), whereupon proteolytic cleavage of its Scc1 subunit causes cohesin to dissociate from chromosomes (Uhlmann et al., 1999). Scc1 cleavage occurs within two related sequence motifs. Mutation of both sequences abolishes cleavage and blocks the separation of sister chromatids. These results demonstrate that Scc1 cleavage is essential for sister chromatid separation. They also raise the possibility that Scc1 cleavage might be the long sought after anaphase trigger.

Scc1's cleavage and dissociation from chromosomes depends both in vitro and in vivo on a protein called Esp1 (Uhlmann et al., 1999). Because of their key role in separating sister chromatids, Esp1 and its homologs in other organisms have been called separins (Funabiki et al., 1996a; Ciosk et al., 1998). For most of the cell cycle, separins are bound by inhibitory proteins called securins (called Pds1 in budding yeast) (Yanagida, 2000). Shortly before the metaphase to anaphase transition, ubiquitination of securin by the anaphase-promoting complex (APC) in conjunction with the Cdc20 protein causes securin t be degraded by the 26S proteosome (Irniger et al., 1995; Cohen-Fix t al., 1996; Funabiki et al., 1996b; Yamamoto et al., 1996). This activates separin

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and enables it to promote Scc1 cleavage and sister separation (Ciosk et al., 1998; Uhlmann et al., 1999).

Previous studies have left unanswered whether separin is itself the protease that cleaves Scc1, whether Scc1 cleavage during metaphase is sufficient to trigger chromosome segregation, and whether separin has functions besides Scc1 cleavage that help orchestrate chromosome movement during anaphase. We show here that separin purified from yeast can cleave pure Scc1 protein prepared from insect cells. We also demonstrate that separin's active site resembles that of the CD clan of cysteine proteases, a group of related proteases that includes caspases. To address whether cleavage of Scc1 is separin's main if not sole function, we replaced one of Scc1's cleavage sites by that of a foreign protease and showed that cleavage by this protease is sufficient to trigger chromosome segregation in metaphase arrested cells.

Results

Mitosis-Specific Phosphorylation of Scc1 Correlates with Efficient Cleavage

In our previous study, Scc1 was presented to crude extracts containing separin in the form of a crude chromatin preparation. To address whether separin is the protease responsible for cleaving Scc1, we needed to prepare a better defined substrate. To do this, we first characterized the state of Scc1 protein at the time of its cleavage. We used a yeast strain whose sole source of the APC activator protein Cdc20 was under control of the galactose inducible *GAL1* promoter. Cells were arrested in metaphase by incubation in medium lacking galactose and subsequently induced to undergo anaphase by readdition of galactose (Uhlmann et al., 1999) (Figure 1A).

Using Western blotting after SDS-PAGE, we noticed that a large fraction of Scc1 prepared from metaphase arrested cells migrated more slowly during electrophoresis than Scc1 from asynchronous cells (Figure 1B). The slower migrating form of Scc1 disappeared as Scc1 cleavage products appeared, whereas the amount of the faster migrating form remained unchanged (Figure 1B, 10 min). This suggests that modification of a fraction of Scc1 during metaphase decreases its electrophoretic mobility and that modified but not unmodified Scc1 might be destined for cleavage during anaphase. Phosphatase treatment of Scc1 from chromatin showed that its decreased mobility when isolated from metaphase cells was due to phosphorylation (Figure 1C).

To address whether Scc1's phosphorylation affects its cleavability in vitro, chromatin from metaphase arrested cells was treated either with phosphatase or with a control buffer. The chromatin was subsequently washed to remove the phosphatase and used as substrate in an Scc1 cleavage reaction. While hyperphosphorylated Scc1 from the control chromatin was efficiently cleaved, Scc1 from the phosphatase treated chromatin was almost completely resistant to cleavage (Figure 1C). This suggests that mitosis-specific phosphorylation might make Scc1 a better substrate for cleavage. However, phosphatase treatm nt of th chromatin might have reduced the phosphorylation of other

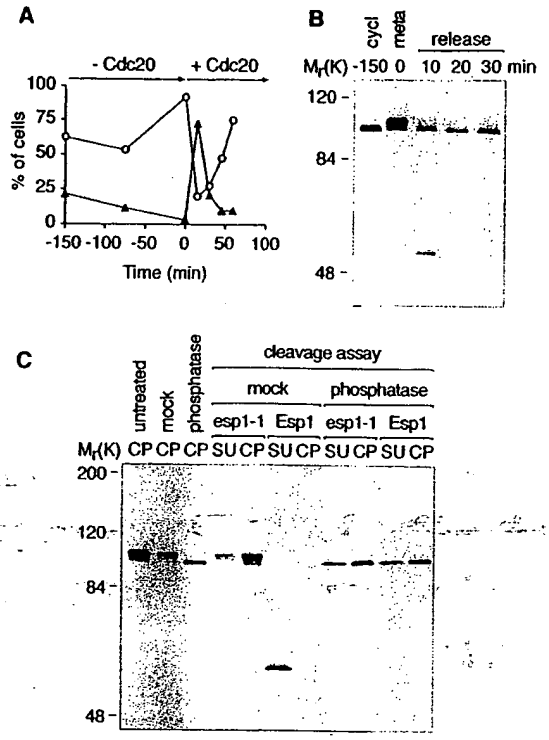

Figure 1. Mitotic Phosphorylation Makes Scc1 a Substrate for Esp1-Dependent Cleavage

(A) Metaphase arrest and release of strain K7677 (*MATa cdc20Δ GAL-CDC20 SCC1-HA3 TetR-GFP TetOs*) was performed as in (Uhlmann et al., 1999). The percentage of cells with Scc1 bound to chromosomes was determined on chromosome spreads (open circles), cells with separated sister chromatids were identified by the appearance of two separated CenV-GFP signals (filled triangles).

(B) SDS-PAGE analysis and Western blot of whole cell extracts prepared from cells at the indicated time points. Scc1-HA3 was detected with monoclonal antibody 16B12 (Babco).

(C) Chromatin was prepared from cells of strain K7677 arrested in metaphase. Chromatin was treated with lambda phosphatase, or mock treated, and used as substrate in an Scc1 cleavage assay as described (Uhlmann et al., 1999). Aliquots of the chromatin preparations (CP), as well as aliquots of the supernatant fractions (SU) and remaining chromatin (CP) after incubation in the cleavage assay were analysed by SDS-PAGE and Western blotting as in (B).

cohesin subunits and/or other chromosomal proteins, which may also have contributed to the reduced Scc1 cleavage.

Phosphorylated Recombinant Scc1 Is a Cleavage Substrate

To establish whether Scc1 can serve as a cleavage substrate when separated from chromatin and other cohesin subunits, we purified Scc1 from insect cells expressing a Flag-epitope-tagged yeast *SCC1* gene from a recombinant baculovirus (Figure 2A). We also purified yeast Scc1 from baculovirus infected insect cells after they had been treated with Okadaic acid. This is a phosphatase inhibitor that induces a metaphase-like stat in which many mitotic proteins are hyperphosphorylated (Kramer et al., 2000). Okadaic acid treatment caused

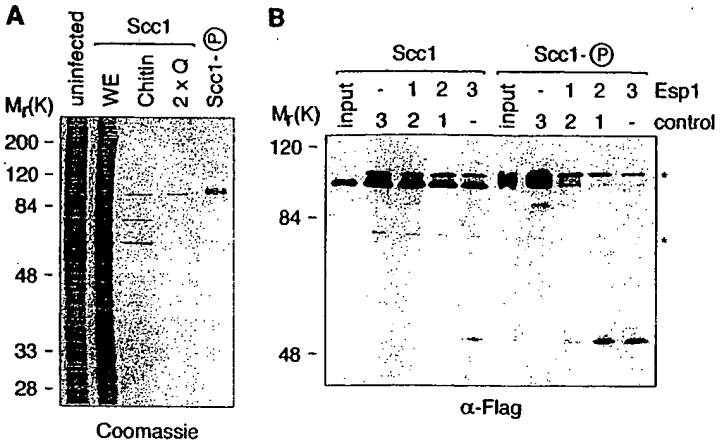

Figure 2. Purified Recombinant Scc1 in a Mitotically Phosphorylated Form Is a Cleavage Substrate

(A) Purification of Scc1 after expression from a recombinant baculovirus in insect cells. Whole cell extracts from uninfected and infected (WE) insect cells were analyzed by SDS-PAGE and staining of the gel with Coomassie brilliant blue. Fractions after chitin affinity chromatography (Chitin) and after two chromatographic steps on a MonoQ column (2 × Q) are shown. The final fraction after purifiction of Scc1 from insect cells treated with Okadaic acid (Scc1-P) is also shown.

(B) Purified Scc1 and Scc1-P were incubated in cell extracts from yeast strain K7287 *(MATα, esp1-1, GAL-ESP1 × 3, TetR-GFP TetOs)*. Extract from a culture that had been induced to express Esp1 for 4 hr before harvesting (Esp1) was mixed with extract from uninduced cells (control) in the indicated ratios. After incubation, aliquots of each reaction were analyzed by SDS-PAGE followed by Western blotting against the Flag epitope at the C terminus of Scc1 using monoclonal antibody M2 (Sigma). Asterisks indicate Flag crossreacting bands present in the yeast cell extracts.

yeast Scc1 to migrate more slowly during SDS-PAGE (Figure 2A) and this was reversed by phosphatase treatment (data not shown). We called this hyperphosphorylated form Scc1-P. Scc1 and Scc1-P were incubated in yeast extracts containing varying amounts of separin (Figure 2B). Some underphosphorylated Scc1 was cleaved by extracts with the highest Esp1 concentration, but most remained uncleaved (Figure 2B, left half). In contrast, all Scc1-P was cleaved by extracts containing intermediate levels of Esp1 separin (Figure 2B, right half). We conclude that soluble recombinant Scc1 can be cleaved in an Esp1-dependent reaction and that mitosis-specific phosphorylation of Scc1 increases its susceptibility to cleavage.

Affinity-Purified Esp1 Separin Cleaves Scc1-P

Scc1 proteolytic activity has thus far only been detected in crude extracts prepared from yeast cells overexpressing Esp1 separin. The protease activity might be due entirely to separin or due to a protease that is merely activated by separin. To distinguish between these two alternatives, we expressed from the *GAL* promoter a version of Esp1 separin containing a chitin binding domain fused to its C terminus and used chitin beads to affinity purify the Esp1 fusion protein from yeast extracts (Figure 3A). This protein was also tagged at its N terminus with a Flag epitope.

Ponceau S staining detected only two abundant polypeptides in the affinity purified material. Western blotting against the Flag epitope showed that the 180 kDa band corresponded to the Esp1 fusion protein (Figure 3A, right panel), whereas mass spectrometry showed that a 70 kDa band was a mixture of 4 members of the Hsp70 family of chaperone proteins (data not shown). Copurification of heat shock proteins indicates that a sizable fraction of the overproduced Esp1 separin might be in a nonnative conformation. It is likely that silver staining would have detected several other proteins in this preparation, albeit at low abundance compared to the Esp1 fusion protein.

Remarkably, Scc1-P was efficiently cleaved when incubated with chitin beads loaded with Esp1 separin but not with beads prepared from uninduced extracts (Figure 3B). Thus, Esp1 separin might indeed be the protease that cleaves Scc1. However, we cannot eliminate at this stage the possibility that the Scc1 cleavage activity is due to a substoichiometric protease that copurifies with separin.

The Conserved Separin Domain Contains a Region that Resembles the Catalytic Site of CD Clan Cysteine Proteases

All known Esp1 separin homologs possess a conserved C-terminal "separin" domain. The recent arrival in ge-

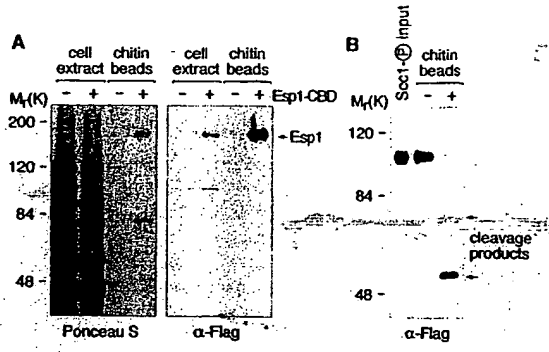

Figure 3. Affinity Purified Esp1 Cleaves Scc1-P

(A) Cell extracts from strain K8965 *(MATα, esp1-1, GAL-Flag-ESP1-CBD × 6, TetR-GFP TetOs)* were prepared without or with induction of Esp1 from the galactose inducible *GAL* promoter. Esp1 was affinity purified on a chitin resin as described in Experimental Procedures. Aliquots of the cell extracts and the chitin beads were analyzed by SDS-PAGE followed by transfer of the gel to a nitrocellulose membrane and Ponceau S staining (left panel). Esp1 was identified by probing the membrane with antibodies against the Flag epitope at the N terminus of Esp1 (right panel).

(B) Scc1-P was added to the chitin beads after the purification procedure and incubated. After incubation, the supernatant above the chitin beads was recovered and analyzed by SDS-PAGE and Western blotting against the Flag epitope on Scc1.

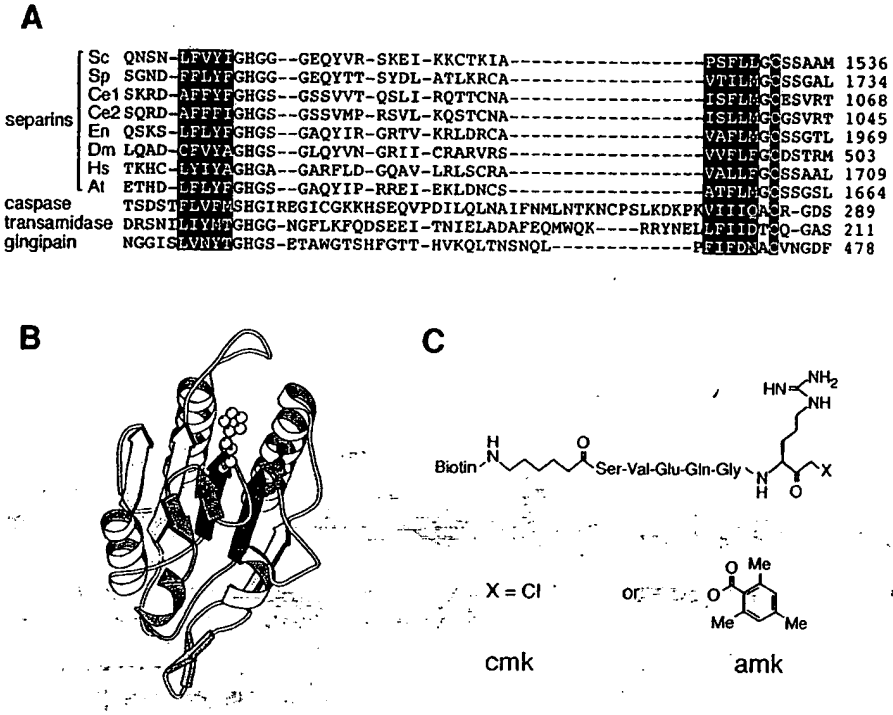

Figure 4. Esp1 and the CD Clan of Cysteine Proteases

(A) Sequence conservation between separins in the region of the proposed proteolytic catalytic site. Sequence alignment in the region of the strictly conserved His and Cys residues is shown together with the sequences of human caspase 1, the transamidase Gpi8, and gingipain. Key to sequences: separins from *S. cerevisae* Esp1 (AAB03897), *S. pombe* Cut1 (A35694), two proteins from *C. elegans* (AAA83576 and the WormPep-Entry CE22098), *E. nidulans* BimB (P33144), *D. melanogaster* (conceptual translation of AC0114811), *H. sapiens* (BAA11482), *A. thaliana* (CAA19812), human caspase 1 (P29466), human Gpi8 (CAA68871), and *P. gingivalis* gingipain (P95493).

(B) The catalytic site of gingipain (Eichinger et al., 1999) is shown as a model for that of separin. The histidine and cysteine catalytic residues are colored yellow and red respectively, whereas hydrophobic parallel β strands on the N-terminal side of these catalytic residues are shown in blue. The polypeptide chain between the two strands is in green. Residues within separins that are proposed to correspond to these structural motifs are marked with the same color scheme in the alignment in A. The figure was drawn with MOLSCRIPT (Kraulis, 1991).

(C) Structure of the chloromethyl ketone (cmk) and acyloxymethyl ketone (amk) derivatives of the Scc1 cleavage site peptide, synthesized as inhibitors against Esp1.

nome databases of several new members of this family have highlighted the most conserved amino acid residues within the separin domain. They include a conserved histidine and cysteine residue, which are hallmarks of cysteine proteases (Figure 4A)(Barrett et al., 1998). These two residues are invariably surrounded by small residues (glycine or serine), and each is preceded by amino acid sequences that are predicted to form hydrophobic β sheets. This pattern is characteristic of cysteine endopeptidases of the CD clan, which includes caspases, legumains, gingipains, clostripains, and GPI-anchor amidases (Chen et al., 1998; Eichinger et al., 1999). In the case of caspase and gingipain, whose crystal structures are known, the two hydrophobic β sheets associate with each other in parallel, bringing the histidine and cysteine residues into juxtaposition to form the catalytic dyad (Figure 3B)(Eichinger et al., 1999). This predicts that the conserved histidine in Esp1 at amino acid position 1505 and the cysteine at position 1531 should be essential for its proteolytic activity (see below).

An Esp1 Inhibitor

If separins are proteases whose mechanism resembles that of caspases, then it should be possible to create peptide based inhibitors (Nicholson et al., 1995; Faleiro et al., 1997) that form a covalent bond with their active site cysteine. Our yeast separin inhibitors were based on the hexapeptide sequence "SVEQGR", which precedes the (more efficiently used) cleavage site in Scc1 at amino acid position 268 (Uhlmann et al., 1999). The C terminus corresponding to the P1 position of the Scc1 cleavage site was modified to a chloromethyl ketone (cmk) or an acyloxymethyl ketone, (amk) (Aplin et al., 1983; Krantz, 1994). Meanwhile, a biotin moiety was linked to the N terminus of the peptide to allow identification of proteins to which the inhibitor might bind (Figure 4C).

Both inhibitors were effective at inhibiting cleavage of Scc1 (presented on yeast chromatin) by crude extracts containing separin (Figure 5). Preincubation with 10 μM Bio-SVEQGR-amk or 100 μM Bio-SVEQGR-cmk inhibited most cleavage activity. We could not detect labeling of any specific protein in the extracts treated with 10 μM Bio-SVEQGR-amk, presumably due to a high background level of naturally biotinylated proteins (data not shown). The same was true for the cmk derivative, whose less specific reactivity might have been responsible for its low r efficiency as a separin inhibitor in crude extracts. Crucially, Bio-SVEQGR-amk failed to inhibit, even at 1 mM, the ability f the TEV protease (Parks et

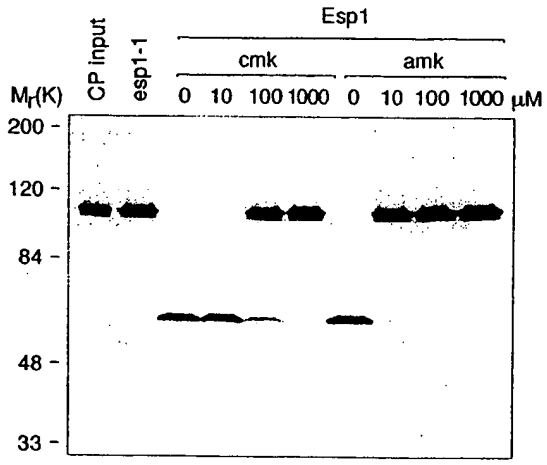

Figure 5. Inhibition of Scc1 Cleavage by the Covalent Inhibitors

An Scc1 cleavage assay was performed as described (Uhlmann et al., 1999), using chromatin prepared from strain K7563 *(MATα, SCC1-HA6)*, arrested in metaphase by treatment with nocodazole, as the substrate. Cell extract used for the reaction from strain K7287 *(MATα, esp1-1, GAL-ESP1-3, TetR-GFP TetOs)* was preincubated with the indicated concentrations of either inhibitor. After the reaction, chromatin and supernatant were not separated again, but an aliquot of the reaction mix was analyzed by SDS-PAGE and Western blotting against the HA epitope on Scc1.

al., 1995), which is also a cysteine protease, to cleave a version of Scc1 engineered to contain a TEV protease recognition site (see below and data not shown). We conclude that Bio-SVEQGR-amk selectively inhibits the Scc1 cleaving protease in crude extracts containing Esp1 separin.

The Effect of Catalytic Dyad Mutations on Protease Activity and Inhibitor Binding

We next mutated the histidine 1505 and cysteine 1531 residues that are predicted to form separin's catalytic dyad (Figure 4A). Both wild-type and the two mutant forms of Esp1 separin were purified after overexpression in yeast. SDS-PAGE analysis followed by Coomassie blue staining (Figure 6A) and Western blotting (Figure 6B) of the material bound to chitin beads showed that they contained similar amounts of wild-type or mutant separin protein. Beads containing wild-type protein but not those containing the mutant proteins fully cleaved Scc1-P (Figure 6C). Underphosphorylated Scc1 isolated from insect cells that had not been treated with Okadaic acid was a poorer substrate (last two lanes in Figure 6C). These results imply that histidine 1505 and cysteine 1531 are crucial for the protease activity associated with Esp1 separin and that phosphorylation of Scc1 facilitates its recognition and/or cleavage. It seems unlikely that two independent single amino acid changes in Esp1 would abolish the copurification of a contaminating protease. Both point mutations also abolished Esp1's ability, when expressed from the *GAL* promoter, to rescue the temperature sensitive lethality of a strain carrying the *esp1-1* mutation (data not shown). This indicates that both histidine 1505 and cysteine 1531 are essential for Esp1 function in vivo.

The Scc1-P cleavage activity associated with wild-type protein was abolished by preincubation of the beads in 10 μM Bio-SVEQGR-amk (Figure 6C). To detect the proteins to which the inhibitor had bound, beads containing either wild-type, H1505A, or C1531A Esp1 were incubated in the presence or absence of 10 μM Bio-SVEQGR-amk. Proteins from the beads were then separated by SDS-PAGE and analyzed by anti-biotin affinity blotting. Trace amounts of a highly abundant biotinylated cellular protein (asterisk in Figure 6D) were detected by this method on untreated beads containing either wild-type or mutant proteins. In the case of wild-type Esp1 and H1505A mutant Esp1, two other proteins with apparent molecular weights of around 180 kDa and 80 kDa were labeled with biotin only upon incubation with Bio-SVEQGR-amk (Figure 6D). Western blotting showed that the 180 kDa protein comigrated with the Flag-tagged Esp1 fusion protein. Neither the 180 kDa protein nor the 80 kDa protein was labeled by the inhibitor when the beads contained C1531A mutant protein. These results suggest that the 180 kDa protein is full-length Esp1 separin, whereas the 80 kDa protein is a cleavage product of Esp1 that lacks its N-terminal Flag epitope tag but contains its C-terminal catalytic cysteine.

The Bio-SVEQGR-amk inhibitor was designed to covalently modify the cysteine within separin's potential active site. The dependence of Esp1 labeling with Bio-SVEQGR-amk on cysteine 1531 is therefore consistent with the assignment of this residue to separin's active site. The histidine residues within the catalytic dyads of cysteine proteases are essential for their action on natural polypeptide substrates but they are dispensable for the binding of these covalent inhibitors. Thus, both the labeling of Esp1 by Bio-SVEQGR-amk and the dependence of this labeling on cysteine 1531 confirm that Esp1 separin is indeed the target of this inhibitor. Esp1 separin must therefore be the protease responsible for Scc1 cleavage.

Scc1 Cleavage Triggers Anaphase

Our finding that Esp1 separin is the Scc1 protease raises the possibility that cleavage of Scc1 might be Esp1's sole function and that this event alone triggers chromosome movement during anaphase. It is also possible that the Esp1 protease has several other important substrates whose cleavage is necessary for chromosome movement. Esp1 might even have activities unrelated to proteolysis, given that its protease domain is situated at the C terminus of a rather large protein.

If cleavage of Scc1 by Esp1 separin were sufficient to trigger anaphase, then cleavage of Scc1 by a protease other than Esp1 should be sufficient to trigger sister chromatid segregation in cells that have been arrested in metaphase. To test this, we took advantage of the fact that Scc1 has two separate cleavage sites, either of which can be mutated without blocking sister chromatid separation as long as the other site remains unchanged (Uhlmann et al., 1999). This enabled us to exchange the cleavage site at position 268 with the recognition sequence for the tobacco etch virus (TEV) protease (Dougherty et al., 1989) (Figure 7A). This Scc1 variant, called Scc1-TEV268, was capable of supporting th proliferation of cells lacking any other form of Scc1. Western blot analysis confirmed that the remaining separin rec-

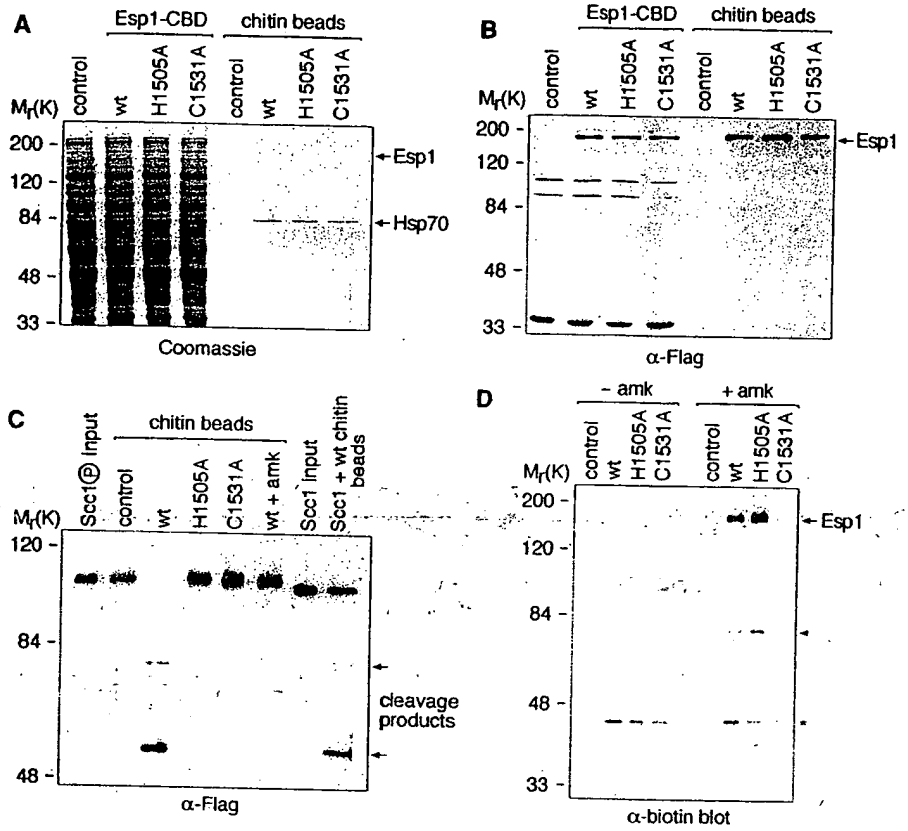

Figure 6. Mutants in the Predicted Esp1 Catalytic Active Site and Affinity Labeling of the Protease.

(A) Cell extracts were prepared from strains K8965 *(MATα, esp1-1, GAL-Flag-ESP1-CBD × 6, TetR-GFP TetOs)* before and after induction, and from strains K8966 *(MATα, esp1-1, GAL-Flag-ESP1-CBD(H1505A) × 6, TetR-GFP TetOs)* and K8967 *(MATα, esp1-1, GAL-Flag-ESP1-CBD(C1531A) × 6, TetR-GFP TetOs)* after induction of Esp1 expression for 5 hr. Esp1 was affinity purified from these extracts as described in Experimental Procedures. Aliquots of the cell extracts and of the material bound to the chitin beads after purification were analyzed by SDS-PAGE followed by staining of the gel with Coomassie brilliant blue.

(B) As (A), but proteins were analyzed by SDS-PAGE followed by Western blotting against the Flag epitope on Esp1. Three Flag crossreacting bands are visible in the uninduced control extract.

(C) Purified recombinant Scc1-P was incubated with affinity purified wild-type and mutant Esp1 on chitin beads. A reaction with Scc1-P incubated with chitin beads after a mock purification from extracts without induced Esp1 is shown (control), as well as a reaction where wild-type Esp1 was treated with the Bio-SVEQGR-amk inhibitor before adding Scc1-P. The last two lanes show a reaction using underphosphorylated Scc1 as the substrate on chitin beads with bound wild-type Esp1.

(D) Control chitin beads, and wild-type and mutant Esp1 bound to chitin beads were incubated without or with addition of 10 μM Bio-SVEQGR-amk. After incubation, proteins bound to the chitin beads were analyzed by SDS-PAGE followed by anti-biotin affinity blot (Falelro et al., 1997).

ognition site at position 180 was cleaved in cycling cells (Figure 7B, cycl).

We next introduced TEV protease (Parks et al., 1995) under control of the inducible *GAL* promoter. Nuclear localization signals were attached to the protease to prevent its accumulation solely in the cytoplasm and myc epitopes were added to detect its expression in yeast. The myc-tagged TEV protease was induced in nocodazole arrested cells containing Scc1-TEV268. The protease accumulated within the nuclei of most cells after two hours and caused cleavage of Scc1-TEV268 at the expected position (Figure 7B). Analysis of chromosome spreads showed that Scc1-TEV268 cleavage was accompanied by its dissociation from chromosomes (Figure 7C). This demonstrates that cleavage of Scc1 is sufficient to induce its dissociation from chromosomes.

Induction of the TEV protease in nocodazole arrested cells expressing only wild-type Scc1 caused neither cleavage nor dissociation from chromosomes (Figure 7C and data not shown).

To address whether cleavage of Scc1 induced by the TEV protease is sufficient to trigger anaphase, we used cells whose sole *CDC20* gene was under control of the *MET3* promoter (Yeong et al., 2000). We arrested these cells in metaphase by transferring them from minimal medium lacking methionine to complete medium containing methionine. Cells accumulated with large buds, replicated chromosomes, and a short bipolar mitotic spindle spanning a single DNA mass at the bud neck. TEV protease synthesis was then induced by the addition of galactose. Induction was l ss efficient than in nocodazole arrested cells but TEV protease neverthe-

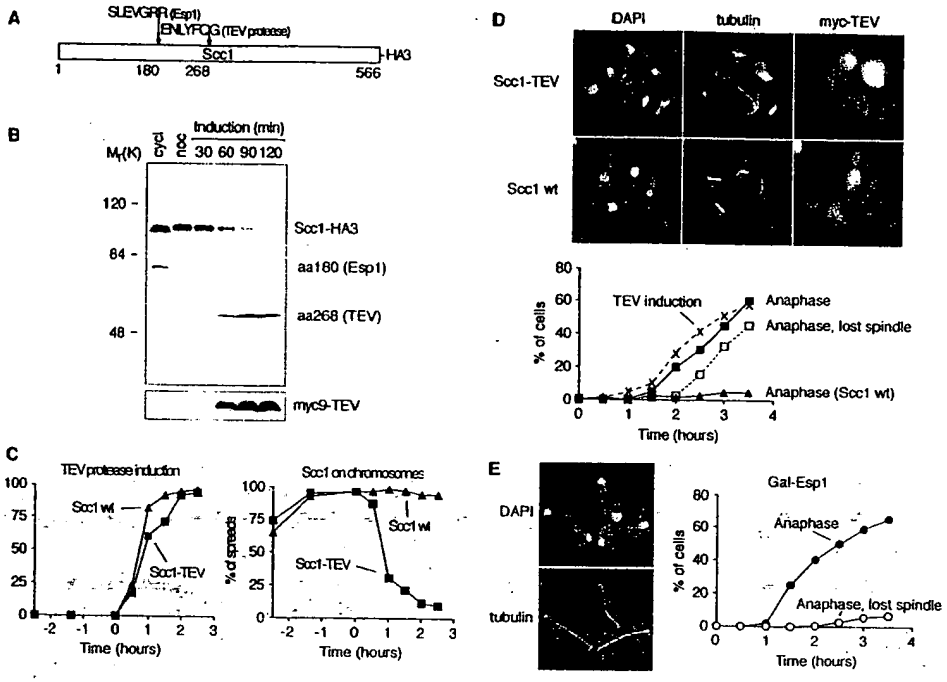

Figure 7. Scc1 Cleavage by TEV Protease Triggers Anaphase

(A) Schematic of the two cleavage sites in Scc1. The wild-type cleavage site at amino acid postion 180, and the TEV protease consensus recognition site that replaces the cleavage site at position 268.

(B) Strain K8758 *(MATα, scc1Δ, SCC1-TEV268-HA3, GAL-NLS-myc9-TEV-NLS2 × 10)* was grown in YEP medium with raffinose and arrested with nocodazole for 2.5 hr. Then, 2% galactose was added to induce TEV protease expression. Aliquots of the culture were harvested at the indicated times and whole cell extract was prepared and analyzed by SDS-PAGE followed by Western blotting against the HA epitope on Scc1.

(C) As (B), but also strain K9127 *(MATα, SCC1-HA3, GAL-NLS-myc9-TEV-NLS2 × 10)* was used in a parallel experiment. TEV protease expression was scored by in situ immunostaining against the myc epitope using monoclonal antibody 9E10. Scc1 association with chromosomes was seen on chromosome spreads stained for the HA epitope on Scc1 using monoclonal antibody 16B12 (Babco).

(D) Cells of strains K9027 *(MATα, MET3-CDC20, scc1Δ, SCC1-TEV268-HA3, GAL-NLS-myc9-TEV-NLS2 × 10, tetOs, tetR)* and K9128 *(MATα, MET3-CDC20, SCC1-HA3, GAL-NLS-myc9-TEV-NLS2 × 10, tetOs, tetR)* were arrested in metaphase as described in Experimental Procedures. At 30 min intervals, samples were retrieved from the culture and processed for immunostaining against tubulin using monoclonal antibody YOL1/34 (Serotec) and against the myc epitope on TEV protease using monoclonal antibody 9E10. DNA was stained with DAPI. The pictures shown are from cells at 3 hr after induction of TEV protease by galactose addition.

(E) As (D), but strain K9129 *(MATα, esp1-1, MET3-CDC20, SCC1-HA3, GAL-Flag-ESP1-CBD × 6, tetOs, tetR)* was used for the experiment.

less accumulated in 40% of the cells within two hours of galactose addition. Remarkably, most cells that had accumulated TEV protease after two hours possessed an elongated anaphase spindle and two equal DNA masses at opposite poles of the cell (Figure 7D, Scc1-TEV). In contrast, cells that had not accumulated TEV protease still possessed short metaphase spindles and a single DNA mass close to the bud neck. In cells with separated DNA masses, sister DNA sequences at the *URA3* locus, which were marked by GFP (Michaelis et al., 1997), were found in opposite halves of the cell (data not shown), indicating that sister chromatids had been properly segregated. We repeated this experiment with cells that expressed wild-type Scc1 and found that cells which had accumulated TEV protease still possessed short spindles and unseparated DNA masses at the bud neck (Figure 7D, Scc1 wt). This shows that cleavage of Scc1 in metaphase arrested cells is sufficient to trigger the movement of sister chromatids to opposite poles. They are presumably pulled there by the mitotic spindle, whose elongation is also triggered by Scc1 cleavage (Figure 7D).

At later time points after TEV protease induction, we observed an increasing number of cells whose elongated mitotic spindles had become weaker or had even disappeared. Such cells had accumulated high levels of TEV protease and had therefore expressed the protease for longer than other cells (Figure 7D). These cells invariably contained two spindle poles at opposite ends of the cell. Figure 7D shows that cells with fully separated DNA masses (with or without anaphase spindles) started to accumulate 60 min after galactose addition, whereas cells with fully separated DNA masses and faint or disassembled anaphase spindles did so 30 min later. These results suggest that TEV protease triggers both chromatid segregation and expansion of pole to pole microtubules but that the highly elongated spindles produced under these circumstances are unstable and break down approximately 30 min after their formation.

Disassembly of the mitotic spindle is not observed in Cdc20 deprived cells which have been permitted to undergo anaphase by deletion of the *PDS1* gen (Shirayama et al., 1999). This suggests that the spindle instability of Cdc20 deprived cells induced to underg ana-

phase by the TEV protease cannot be due to the lack of Cdc20 function per se and suggests that it might be due to the lack of separin activity. To test whether spindles would be more stable in the presence of Esp1 separin, we analyzed spindle stability in Cdc20 depleted cells induced to undergo anaphase by expressing high levels of Esp1 from the *GAL* promoter. Induction of Esp1 triggered anaphase in a large fraction of cells and did so somewhat more rapidly than the TEV protease. Crucially, the extended anaphase spindles produced by Esp1 persisted even after long periods in all cells that had fully segregated their chromosomes (Figure 7E). We conclude that while cleavage of Scc1 in metaphase triggers chromatid segregation, it might not be sufficient to bring about a stabilization of the mitotic spindle that is necessitated by the movement of spindle poles to opposite ends of the cell. We cannot at this stage completely exclude the possibility that destabilization of anaphase spindles is an artifact of TEV protease expression. However, this seems unlikely because cells containing wild-type Scc1 proliferate normally even when high levels of TEV protease are continuously expressed (data not shown).

Discussion

Our previous experiments demonstrated that Scc1 cleavage is crucial for sister chromatid separation at the onset of anaphase. We also described a crude cell-free system that recapitulated cleavage of Scc1 and its dissociation from chromosomes (Uhlmann et al., 1999) and showed that both events were dependent on Esp1 separin. However, these experiments neither identified the protease responsible for Scc1 cleavage nor addressed whether cleavage actually triggers anaphase. The experiments described here show that Esp1 separin is the Scc1 protease and that Scc1 cleavage is the event that triggers the segregation of sister chromatids to opposite poles of the cell at the metaphase to anaphase transition.

Reconstitution of Scc1 Cleavage with Purified Components

Identification of the Scc1 protease required two separate tasks: first to prepare a fully defined substrate and second to purify the protease. We found that pure yeast Scc1 is a substrate for Esp1-dependent cleavage as long as it is hyperphosphorylated either during mitotic arrest in yeast cells or by the treatment of insect cells with Okadaic acid. Though our experiments do not directly address the physiological significance of Scc1 phosphorylation, they nevertheless raise the possibility that Scc1 cleavage is controlled by its cell-cycle-regulated phosphorylation as well as by cell cycle dependent destruction of the Esp1 inhibitor Pds1 securin.

Our identification of the Scc1 protease started from the premise that Scc1 cleavage is an Esp1-dependent reaction. We therefore set out to purify Esp1 and to characterize its activity. Our first attempts yielded virtually homogeneous Esp1 preparations which lacked any proteolytic activity on their own and also failed to stimulate Scc1 cleavage when added back to yeast cell extracts (data not shown). We found that the Scc1 cleaving activity from yeast cell extracts overexpressing Esp1 was unstable, with a half-life of a few hours. We therefore used a rapid single step purification scheme in which Esp1 fused to a chitin binding domain was bound to a chitin resin. Remarkably, such beads efficiently cleaved pure phosphorylated Scc1. This result suggested that Esp1 separin might alone be capable of cleaving Scc1 but it did not exclude the possibility that a substoichiometric protease bound to Esp1 was responsible for the proteolytic activity. The final line of argument that Esp1 is indeed the Scc1 protease was inspired by the discovery that separins contain a highly conserved domain at their C terminus that resembles the catalytic dyad of an established class of cysteine proteases.

Separins and the CD Clan of Cysteine Proteases

Sequence similarity between separins from different organisms is largely confined to a C-terminal region spanning some 400 residues, with the highest conservation within the 100 C-terminal residues. When the conserved amino acid pattern hhhhx[GS]HGx(4)hx(14,32)hhx[GA]Cx[GS] (h indicates a hydrophobic residue; x indicates any residue; letters in brackets indicate alternative residues; numbers in parentheses indicate the number of residues in an interval; and numbers separated by a comma mean "from–to") was used to search the complete protein sequence database, 175 sequences were retrieved, all of them representing either the separin family or the CD clan of cysteine proteases. Some of the latter were missed, but no false-positives were detected. A notable difference between separins and the CD clan proteases is the much shorter distance that separates the catalytic histidine and cysteine in the former. However, because the length, sequence, and arrangement of structural elements within this spacer are variable within the CD clan itself, this difference is not incompatible with the hypothesis that separins contain a cysteine protease domain that is structurally similar and evolutionarily related to CD clan proteases. It is remarkable that sequence-specific proteases of the same fold and likely common origin are involved in a crucial aspect of chromosome mechanics and in the control of programmed cell death.

As demanded by this model, the cysteine and histidine residues predicted to be part of separin's active site, were found to be essential for Esp1's proteolytic activity. There is one other cysteine residue that is conserved in the separin domains of most species (cysteine 1556 in Esp1), except for *Arabidopsis* and *Aspergillus*, where it is replaced by a serine. This residue might also qualify as a putative catalytic amino acid. However, mutation of cysteine 1556 to alanine had no effect on Esp1's protease activity (data not shown). We therefore conclude that histidine 1505 and cysteine 1531 likely form Esp1's catalytic dyad.

Inspired by separin's similarity to caspases, we devised peptide based inhibitors that were designed to bind covalently to the active site cysteine of the Scc1 protease. Modification of the C-terminal arginine in the hexapeptide SVEQGR, which precedes the second Scc1 cleavage site, to an acyloxymethyl ketone created a potent and specific inhibitor of the Scc1 cleavage activity associated with purified Esp1. The inhibitor bound

covalently to full length Esp1 and to a minor Esp1 cleavage product but to no other protein in our purified Esp1 preparations. Furthermore, this binding was dependent on the proposed active site cysteine 1531. These results imply that Esp1 is the sole target of our inhibitor and that Esp1 must therefore be the Scc1 protease. It will nevertheless be important to confirm the presence of the CD clan like protease domain within separins by atomic resolution structural studies of Esp1 and its homologs.

Triggering Chromosome Movement in Anaphase

It has been a longstanding question what initiates the segregation of sister chromatids to opposite poles of the cell at the metaphase to anaphase transition. Activation of separin through destruction of their inhibitory securin chaperone by the APC is clearly a crucial step. Our previous finding that Scc1 cleavage is essential for sister chromatid separation together with the discovery that separin is the protease responsible for Scc1 cleavage suggest that Scc1 cleavage alone could trigger anaphase. To test this, we engineered a yeast strain in which one of Scc1's cleavage sites was replaced by that of the foreign TEV protease. Remarkably, induction of TEV protease in cells arrested in metaphase due to Cdc20 depletion was sufficient to cleave Scc1, to cause its dissociation from chromosomes, and to trigger sister chromatid separation and movement to opposite poles of the cell.

The TEV protease experiment also demonstrates that the yeast mitotic spindle is prepared to drive spindle poles to opposite halves of the cell during metaphase but that it is prevented from doing so by cohesin, connecting sister chromatids. To determine more precisely whether the kinetics of spindle extension due to a TEV protease-induced anaphase are similar to that in wild-type cells, it will be necessary to visualize these movements in real time. It is, however, unclear whether accumulation of TEV protease within nuclei following galactose induction is sufficiently instantaneous to make a meaningful comparison at the moment. Remarkably, expression of the TEV protease in yeast cells was harmless to wild-type cells. Thus, the introduction of TEV protease recognition sites to other proteins could also be used to study the consequences of their deliberate cleavage. This technique might be useful also in other organisms and particularly for applications where specific cleavage reactions are involved, e.g., signaling via the notch pathway (Blaumueller et al., 1997).

We noticed that the mitotic spindles of cells which had been induced to undergo anaphase by TEV protease were thinner than normal anaphase spindles and frequently broke after chromosome segregation. This phenotype was not observed when anaphase was triggered by inducing high levels of Esp1 in metaphase arrested cells. This suggests that separin might have a second function besides cleaving Scc1: to stabilize mitotic spindles during anaphase. It has previously been suggested that separin might have a role in spindle function on the basis of its association with mitotic spindles during anaphase (Funabiki et al., 1996a; Ciosk et al., 1998; Kumada et al., 1998). Whether Esp1 performs this function by cleaving other proteins or by a different mechanism remains to be investigated. It is even conceivable that Scc1 cleavage products have a role in anaphase spindle stabilization and that the products produced by TEV protease cleavage are altered in this regard.

There are good reasons to believe that proteolytic cleavage of cohesins by separin might trigger anaphase in all eukaryotic organisms. The catalytic site of yeast Esp1 is conserved in all known separins. Furthermore, an immunopurified Esp1 fraction from human cells posesses Scc1 cleavage activity and Scc1 is both cleaved and disappears from centromeres at the metaphase to anaphase transition in human cells (Waizenegger et al., 2000 [this issue of *Cell*]). Given their conservation and similarity to caspases, we suggest that separins might be better known as "chromatid separases" (Ostergren and Anderson, 1973), or simply "separases".

Experimental Procedures

Dephosphorylation of Scc1 on Chromatin

A crude chromatin fraction containing Scc1 tagged with HA epitopes was obtained as described (Liang and Stillman, 1997). Chromatin obtained from 50 μl cell lysate was resuspended in 200 μl phosphatase buffer (50 mM Tris/HCl [pH 7.5], 100 mM KCl, 0.1 mM EDTA, 2 mM $MnCl_2$, 5 mM dithiothreitol, 0.01% Brij 35, and 0.1% Triton X-100), and 2000 units lambda protein phosphatase (New England Biolabs) was added. Incubation was for 12 min at 30°C, then the chromatin was recovered by centrifugation and washed once before it was used for the Scc1 cleavage assay as described (Uhlmann et al., 1999). To exclude that the effect observed after phosphatase treatment of the chromatin was due to residual amounts of phosphatase carried into the subsequent cleavage reaction, a control reaction was carried out in which 200 units of lambda phosphatase were added to the cell extract. This had no effect on the efficiency of Scc1 cleavage in the assay.

Purification of Scc1 Expressed in Baculovirus Infected Insect Cells

The Scc1 coding sequence was cloned into the baculovirus transfer vector pFastBac1 (Gibco Life Technologies). At the C terminus, a Flag epitope tag was added followed by a cassette containing the yeast VMA intein and a chitin binding domain (New England Biolabs). Recombinant baculoviruses were obtained, and HiFive insect cells (Invitrogen) grown in monolayers to confluency were infected at a multiplicity of infection of 2. To obtain metaphase-like phosphorylation, 0.1 μM Okadaic acid was added 40 hr after infection. Forty three hours after infection, cells were harvested. Cytoplasmic and nuclear extracts were obtained as described (Cai et al., 1996). When Scc1 was purified after treatment of the cells with Okadaic acid, 5 mM NaF and 5 mM sodium pyrophosphate was added to the extraction buffer. Combined cytoplasmic and nuclear extracts from cells in 10 T250 flasks (8 ml, 100 mg protein) were loaded onto 1 ml bead volume of chitin beads (New England Biolabs), equilibrated in buffer H_{200} (50 mM HEPES/KOH [pH 7.5], 200 mM NaCl, 1.5 mM $MgCl_2$, and 0.01% Nonidet P-40). Loading was for 2 hr by rocking at 4°C. The chitin resin was then washed in a column with 20 column volumes each of buffer H_{600}, H_{1000}, and H_{200} (index indicates the concentration of NaCl). The column was then flushed with 3 column volumes of buffer H_{200} containing 50 mM dithiothreitol and closed for cleavage of the intein overnight. The eluate containing Scc1 released from the column was directly loaded onto a MonoQ HR 5/5 column (Amersham Pharmacia), and the column was developed with a gradient from H_{200} to H_{1000} in 10 ml. Fractions containing Scc1 were detected by SDS-PAGE followed by Coomassie staining and Western blotting against the Flag epitope. Pooled fractions were diluted to 200 mM NaCl by adding buffer H_0, and chromatography on the MonoQ column was repeated. This step was necessary to remove traces of a contaminating protein that copurified with Scc1 and was identified as Hsp70 from the insect host cells by mass spectrometry. The final eluate was dialyzed against buffer H_{100} and concentrated by

ultrafiltration. This protocol yielded 25 μg of purified Scc1 or purified metaphase-like phosphorylated Scc1.

Cleavage of Purified Scc1 in Yeast Extracts

Scc1 cleavage-competent yeast extracts after overexpression of Esp1 and control extracts were obtained as described (Uhlmann et al., 1999). Six microliters (20 ng/μl) purified Scc1 or Scc1-P in H_{100} was added to 12 μl of control or Esp1 containing extracts, or to mixtures at different rations between these extracts. Incubation was for 10 min at 25°C. The reaction was stopped by adding SDS-PAGE loading buffer, and aliquots were analyzed by SDS-PAGE and Western blotting against the Flag epitope.

Overexpression and Purification of Esp1 from Yeast Cells

The Esp1 coding sequence was cloned under control of the *GAL* promoter into the yeast vector YIp204 (Gietz and Sugino, 1988). At the N terminus of the protein, a Flag epitope tag was added; at the C terminus a chitin binding domain was added (New England Biolabs). Site directed mutagenesis of the residues His1505, Cys1531, and Cys1556 to alanine was performed using PCR. The linearized vectors were integrated into the yeast genome at the *TRP1* locus, and transformants were identified by Southern blotting that had integrated 6–8 copies of the respective constructs. Two-hundred milliliter cultures of these strains were grown in YEP medium with 2% raffinose as the carbon source (Rose et al., 1990) at 25°C. At a density of 2.5×10^6 cells/ml, the *GAL* promoter was induced by the addition of 2% galactose for 5 hr before cells were harvested. One culture was left uninduced as a control for the purification. Cells were resuspended in 6 ml 100 mM PIPES/KOH (pH 9.3), 10 mM dithiothreitol, and 0.1% sodium azide, and incubated at room temperature for 10 min. Cell were collected again by centrifugation and resuspended in 4 ml of 50 mM potassium phosphate (pH 7.5), 0.6 M sorbitol, 0.5 mM $MgCl_2$, and 10 mM dithiothreitol. Zymolase T-100 (ICN Biochemicals) was added to a final concentration of 40 μg/ml and spheroplasting was at 37°C for 10 min. Cells were collected again, washed in 2 ml of 50 mM HEPES/KOH (pH 7.5), 100 mM KCl, 2.5 mM $MgCl_2$, and 0.4 M Sorbitol, and resuspended in 350 μl buffer FB (50 mM HEPES/KOH [pH 7.5], 250 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM dithiothreitol, plus protease inhibitors as described [Liang and Stillman, 1997]). Cells were lysed by the addition of 0.25% Trition X-100 for 3 min on ice, and the lysate was cleared by centrifugation. The extracts were loaded onto 50 μl bead volume of chitin beads and equilibrated in buffer FBX (FB plus 0.05% Triton X-100) for 2 hr on a rotating wheel. Beads were transferred into a chromatography column and washed with 5 ml each of FBX, FBX_{1000}, and FBX_{100}, containing 1 M and 100 mM NaCl, respectively. Beads were then resuspended in a total volume of 100 μl FBX_{100} and 20 μl aliquots of the suspension were distributed. Beads were sedimented by gravity and 10 μl supernatant was retrieved and discarded. For detection of purified Esp1, 10 μl of SDS-PAGE loading buffer was added to the beads, boiled for 5 min, and 10 μl of the SDS eluate was loaded for SDS-PAGE.

Scc1 Cleavage by Esp1 Bound to Chitin Affinity Beads

Ten microliters (20 ng/μl) of purified Scc1 or Scc1-P in FBX_{100} supplemented with 0.1 mg/ml BSA was added to 10 μl of chitin beads obtained as described above. The reaction was incubated for 15 min at 25°C with continuous shaking. Beads were sedimented by brief centrifugation and 10 μl supernatant was retrieved and loaded for SDS-PAGE. In experiments where the peptide inhibitor was used, 10 μl of a 20 μM dilution of the inhibitor (see below) was added to 10 μl of chitin beads and incubated 10 min at 25°C. Then, 10 μl of supernatant was retrieved again, and Scc1 was added and incubated as above. To detect the proteins on the beads labeled by the inhibitor, the beads were boiled with 10 μl of SDS-PAGE loading buffer and the eluate was loaded for SDS-PAGE. After electrophoresis, the gel was transferred to a nitrocellulose membrane and biotinylated proteins were detected as described (Faleiro et al., 1997).

Sequence Analysis

Searches of the nonredundant protein sequence database and of nucleotide sequence databases at the National Center for Biotechnology Information (NIH, Bethesda) were performed using the PSI-BLAST program (Altschul et al., 1997). Additional database searches combining BLASTP with a pattern analysis were performed using the PHI-BLAST program (Zhang et al., 1998). Database screening for amino acid patterns was performed using the PATTINPROT program at the NPS2 server (Combet et al., 2000). Multiple sequence alignment construction and analysis, with statistical evaluation of the significance of motif conservation, were performed using the GIBBS sampling option of the MACAW program (Schuler et al., 1991; Neuwald et al., 1995). Multiple-alignment-based protein secondary structure prediction was performed using the PHD (Rost and Sander, 1994) and PSIPRED (Jones, 1999) programs.

Peptide Based Covalent Inhibitors for Esp1-Dependent Scc1 Cleavage

A biotinylated peptidyl chloromethyl ketone (cmk) and a peptidyl (2,4,6-trimethylbenzoyloxy)methyl ketone (acyloxymethyl ketone, amk) of the sequence Ser-Val-Glu-Gln-Gly-Arg were synthesized. The side chain protected peptide fragment Biotinyl-6-aminohexanoyl-Ser-Val-Glu-Gln-Gly-OH was obtained by solid phase synthesis on an acid sensitive 2-chlorotrityl resin. H-Arg(Z)2-cmk or H-Arg(Z)2-amk fragments were coupled in solution (Aplin et al., 1983; Krantz, 1994), and the final peptides were deprotected by a TFA-trifluoromethanesulfonic acid-thioanisole mixture (Yaima and Fujii, 1981). The compounds were purified to homogeneity by preparative reversed phase HPLC. One hundred millimolar stock solutions of these inhibitors were prepared in DMSO and stored at −80°C, dilutions were prepared in 50 mM HEPES/KOH (pH 7.5), 100 mM KCl, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol. To assess the effect of the inhibitors on Esp1-dependent Scc1 cleavage, a standard cleavage assay was performed as described (Uhlmann et al., 1999), but the cell extracts used for the cleavage reaction were preincubated for 10 min at 25°C with the inhibitors.

Cleavage of Scc1 by TEV Protease and Esp1 In Vivo

The sequence Ser-Val-Glu-Gln-Gly-Arg-Arg around the cleavage site in Scc1 at amino acid position 268 was changed into the consensus recognition sequence for TEV protease Glu-Asn-Leu-Tyr-Phe-Gln-Gly, (Dougherty et al., 1989) by site-directed mutagenesis. The resulting modified *SCC1-TEV268* gene was cloned together with 1150 nt of upstream promoter sequence into YIp211 (Gietz and Sugino, 1988), and a triple HA epitope tag was added at the C terminus. This *SCC1* construct was integrated at the *LEU2* locus, and correct integration was confirmed by Southern blotting. The endogenous copy of *SCC1* was then disrupted by replacement with the *HIS3* marker gene (Wach et al., 1994). The TEV protease coding sequence was cloned into YIp204 (Gietz and Sugino, 1988) under control of the *GAL* promoter. At the N terminus, the consensus sequence of the SV40 nuclear localization signal (Kalderon et al., 1984) was added together with a tandem repeat of nine myc epitopes. At the C terminus, a repeat of two SV40 nuclear localization signals was added. The resulting construct was integrated into the yeast genome at the *TRP1* locus, and strains with 10 copies of the vector integrated were selected by Southern blot analysis (integrated into the strain containing Scc1-TEV268 only: strain K8758, or containing wild-type Scc1: strain K9127). A strain containing the sole source of Cdc20 under control of the *MET3* promoter was constructed by integrating a modified version of plasmid pUS1278 (Yeong et al., 2000) into strain K7100, containing GFP marked chromosome V (Michaelis et al., 1997). The centromere and ARS region of pUS1278 were replaced by a fragment spanning nucleotides −806 to −212 upstream of *CDC20*. Linearization and integration of this plasmid at the *CDC20* locus results in replacement of the *CDC20* promoter with the *MET3* promoter (Strain K9022). Strains K8758 and K9022 were crossed to yield strain K9027 used for the experiments. A control strain (K9128) was constructed similarly, but only wild-type Scc1 was present in the cells, tagged at the C terminus with a triple HA epitope (Knop et al., 1999). The strain for induction of Esp1 from the *GAL* promoter in metaphase arrest due to depletion of Cdc20 was obtained by crossing strain K8965 (see above) with K9022. For arrest of *MET3-CDC20* cells in metaphase, cells were grown in synthetic minimal medium lacking methionine (Rose et al., 1990) at 23°C. Cells were filtered and transferred into YEP medium containing 2% raffinose supplemented with 2 mM methionine for

arrest in metaphase for 2.5 hr. Then, 2% galactose was added to induce expression of TEV protease or Esp1. The metaphase arrest due to Cdc20 depletion was stable until at least 4 hr after all cells had reached metaphase. In the case of overexpression of Esp1 in the metaphase arrest, a small percentage of cells (less than 20%) formed additional buds after 3.5 hr, probably because cyclin-dependent kinases were partly inactivated under these conditions (Tinker-Kulberg and Morgan, 1999). Cytokinesis or rereplication of the DNA, however, did not occur during the time of observation.

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References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. *25*, 3389–3402.

Aplin, R.T., Christiansen, J., and Young, G.T. (1983). Amino-acids and peptides, part 48. Synthesis of bradykinyl-chloromethane. Int. J. Peptdide Protein Res. *21*, 555–561.

Barrett, A.J., Rawlings, N.D., and Woessner, J.F. eds. (1998). Handbook of proteolytic enzymes (London, Academic Press).

Blaumueller, C.M., Qi, H., Zagouras, P., and Artavanis-Tsakonas, S. (1997). Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. Cell *90*, 281–291.

Cai, J., Uhlmann, F., Gibbs, E., Flores-Rozas, H., Lee, C.-G., Phillips, B., Finkelstein, J., Yao, N., O'Donnell, M., and Hurwitz, J. (1996). Reconstitution of human replication factor C from its five subunits in baculovirus-infected insect cells. Proc. Natl. Acad. Sci. USA *93*, 12896–12901.

Chen, J.M., Rawlings, N.D., Stevens, R.A., and Barrett, A.J. (1998). Identification of the active site of legumain links it to caspases, clostripain and gingipains in a new clan of cysteine endopeptidases. FEBS Lett. *441*, 361–365.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M., and Nasmyth, K. (1998). An Esp1/Pds1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. Cell *93*, 1067–1076.

Ciosk, R., Shirayama, M., Shevchenko, A., Tanaka, T., Toth, A., Shevchenko, A., and Nasmyth, K. (2000). Cohesin's binding to chromosomes depends on a separate complex consisting of Scc2 and Scc4 proteins. Mol. Cell *5*, 1–20.

Cohen-Fix, O., Peters, J.-M., Kirschner, M.W., and Koshland, D. (1996). Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. Genes Dev. *10*, 3081–3093.

Combet, C., Blanchet, C., Geourjon, C., and Deléage, G. (2000). NPS@: network protein sequence analysis. Trends Biochem. Sci. *25*, 147–150.

Dougherty, W.G., Cary, S.M., and Parks, T.D. (1989). Molecular genetic analysis of a plant virus polyprotein cleavage site: a model. Virology *171*, 356–364.

Eichinger, A., Beisel, H.G., Jacob, U., Huber, R., Medrano, F.J., Banbula, A., Potempa, J., Travis, J., and Bode, W. (1999). Crystal structure of gingipain R: an Arg-specific bacterial cysteine proteinase with a caspase-like fold. EMBO J. *18*, 5453–5462.

Faleiro, L., Kobayashi, R., Fearnhead, H., and Lazebnik, Y. (1997). Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. EMBO J. *16*, 2271–2281.

Funabiki, H., Kumada, K., and Yanagida, M. (1996a). Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. EMBO J. *15*, 6617–6628.

Funabiki, H., Yamano, H., Kumada, K., Nagao, K., Hunt, T., and Yanagida, M. (1996b). Cut2 proteolysis required for sister-chromatid seperation in fission yeast. Nature *381*, 438–441.

Gietz, R.D., and Sugino, A. (1988). New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six base pair restriction sites. Gene *74*, 527–534.

Griffin, D.K. (1996). The incidence, origin, and etiology of aneuploidy. Int. Rev. Cytol. *167*, 263–296.

Guacci, V., Koshland, D., and Strunnikov, A. (1997). A direct link between sister chromatid cohesion and chromosome condensation revealed through analysis of MCD1 in S. cerevisiae. Cell *91*, 47–57.

Imiger, S., Piatti, S., Michaelis, C., and Nasmyth, K. (1995). Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. Cell *81*, 269–278.

Jones, D.T. (1999). Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. *292*, 195–202.

Kalderon, D., Roberts, B.L., Richardson, W., and Smith, A. (1984). A short amino acid sequence able to specify nuclear location. Cell *39*, 499–509.

Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K., and Schiebel, E. (1999). Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast *15*, 963–972.

Kramer, E.R., Scheuringer, N., Podtelejnikov, A.V., Mann, M., and Peters, J.-M. (2000). Mitotic Regulation of the APC Activator Proteins CDC20 and CDH1. Mol. Biol. Cell *11*, 1555–1569.

Krantz, A. (1994). Peptidyl (Acyloxy)methanes as Quiescent Affinity Labels for Cysteine Proteinases. Methods Enzymol. *244*, 656–671.

Kraulis, P.J. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J. Appl. Crystallog. *24*, 946–958.

Kumada, K., Nakamura, T., Nagao, K., Funabiki, H., Nakagawa, T., and Yanagida, M. (1998). Cut1 is loaded onto the spindle by binding to Cut2 and promotes anaphase spindle movement upon Cut2 proteolysis. Curr. Biol. *8*, 633–641.

Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1997). Genetic instability in colorectal cancers. Nature *386*, 623–627.

Liang, C., and Stillman, B. (1997). Persistent initiation of replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. Genes Dev. *11*, 3375–3386.

Losada, A., Hirano, M., and Hirano, T. (1998). Identification of Xenopus SMC protein complexes required for sister chromatid cohesion. Genes Dev. *12*, 1986–1997.

Mazia, D. (1961). Mitosis and the Physiology of Cell Division, Vol III (New York, Academic Press).

Michaelis, C., Ciosk, R., and Nasmyth, K. (1997). Cohesins: Chromosomal proteins that prevent premature separation of sister chromatids. Cell *91*, 35–45.

Mole-Bajer, J. (1958). Cine-micrographic analysis of C-mitosis in endosperm. Chromosoma *9*, 332–358.

Nasmyth, K., Peters, J.-M., and Uhlmann, F. (2000). Splitting the chromosome: cutting the ties that bind sister chromatids. Science *288*, 1379–1384.

Neuwald, A.F., Liu, J.S., and Lawrance, C.E. (1995). Gibbs motif sampling: detection of bacterial outer membrane protein repeats. Protein Sci. *4*, 1618–1632.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., et al. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature *376*, 37–43.

Ostergren, G., and Anderson, L. (1973). Staining the Kinetochore

in Plant Chromosomes, Vol *23*, T. Caspersson and L. Zech, eds. (Stockholm, Nobel Foundation).

Parks, T.D., Howard, E.D., Wolpert, T.J., Arp, D.J., and Dougherty, W.G. (1995). Expression and purification of a recombinant tobacco etch virus Nla proteinase: biochemical analyses of the full-length and a naturally occuring truncated proteinase form. Virology *210*, 194–201.

Rose, M.D., Winston, F., and Hieter, P. (1990). Laboratory Course Manual for Methods in Yeast Genetics, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Rost, B., and Sander, C. (1994). Combining evolutionary information and neural networks to predict protein secondary structure. Proteins *19*, 55–72.

Schuler, G.D., Altschul, S.F., and Lipman, D.J. (1991). A workbench for multiple alignment construction and analysis. Proteins *9*, 180–190.

Shirayama, M., Toth, A., Galova, M., and Nasmyth, K. (1999). APC^{Cdc20} promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. Nature *402*, 203–207.

Tinker-Kulberg, R.L., and Morgan, D.O. (1999). Pds1 and Esp1 control both anaphase and miotic exit in normal cells and following DNA damage. Genes Dev. *13*, 1936–1949.

Toth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleifer, A., and Nasmyth, K. (1999). Yeast Cohesin complex requires a conserved protein, Eco1p (Ctf7), to establish cohesion between sister chromatids during DNA replication. Genes Dev. *13*, 320–333.

Uhlmann, F., and Nasmyth, K. (1998). Cohesion between sister chromatids must be established during DNA replication. Curr. Biol. *8*, 1095–1101.

Uhlmann, F., Lottspeich, F., and Nasmyth, K. (1999). Sister chromatid separation at anaphase onset is triggered by cleavage of the cohesin subunit Scc1p. Nature *400*, 37–42.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast *10*, 1793–1808.

Waizenegger, I.C., Hauf, S., Meinke, A., and Peters, J.-M. (2000). Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. Cell *103*, this issue, 399–410.

Yaima, H., and Fujii, N. (1981). Studies on peptides. 103. Chemical synthesis of a crystalline protein with the full enzymatic activity of Ribonuclease A. J. Am. Chem. Soc. *103*, 5867–5871.

Yamamoto, A., Guacci, V., and Koshland, D. (1996). Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathways. J. Cell Biol. *133*, 99–110.

Yanagida, M. (2000). Cell cycle mechansims of sister chromatid separation; roles of Cut1/separin and Cut2/securin. Genes Cells *5*, 1–8.

Yeong, F.M., Lim, H.H., Padmashree, C.G., and Surana, U. (2000). Exit from mitosis in budding yeast: biphasic inactivation of the Cdc28-Clb2 mitotic kinase and the role of Cdc20. Mol. Cell *5*, 501–511.

Zhang, Z., Schaffer, A.A., Miller, W., Madden, T.L., Lipman, D.J., Koonin, E.V., and Altschul, S.F. (1998). Protein sequence similarity searches using patterns as seeds. Nucleic Acids Res. *26*, 3986–3990.

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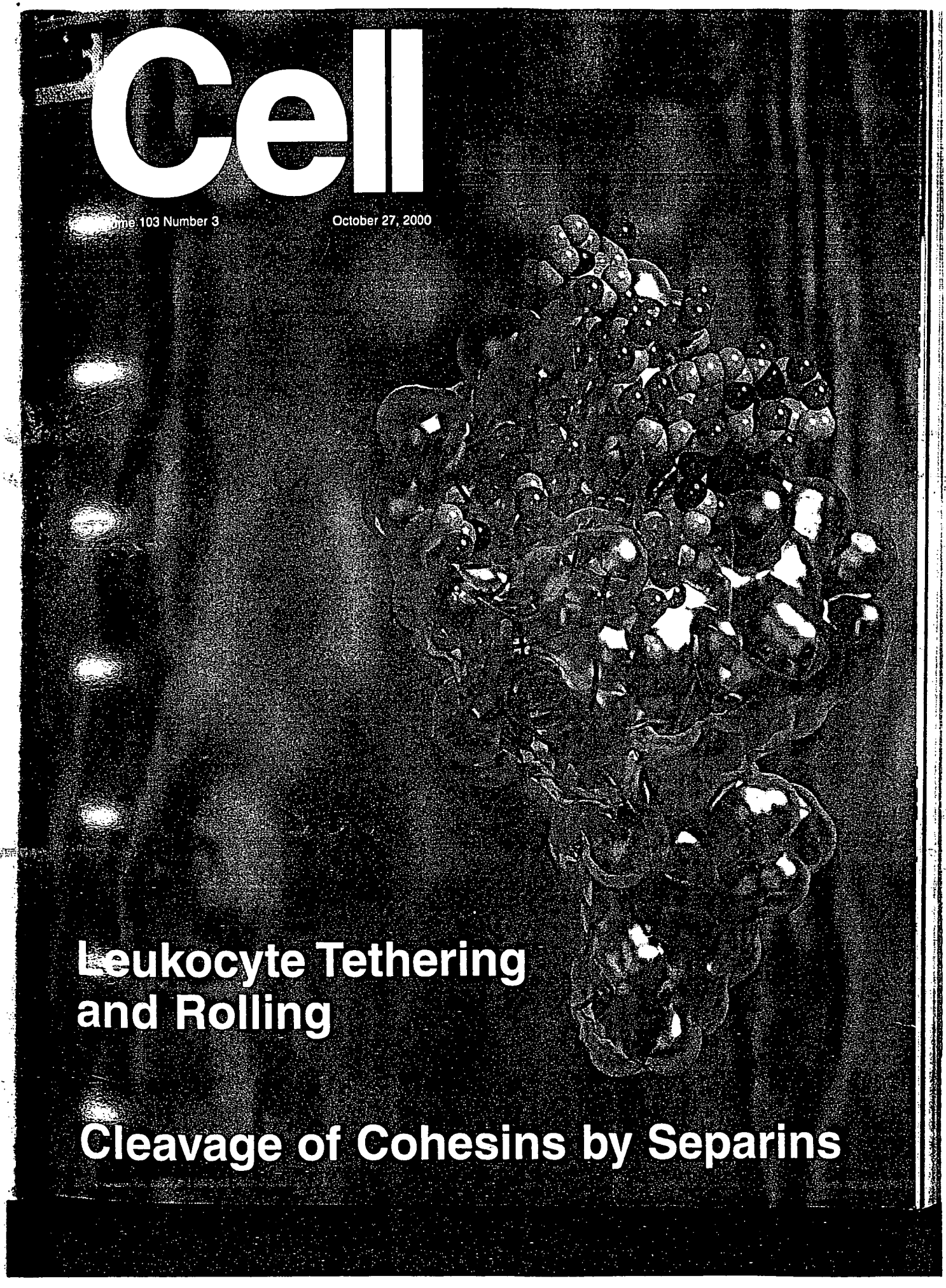

Leukocyte Tethering and Rolling

Cleavage of Cohesins by Separins

Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1

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Cohesion between sister chromatids is established during DNA replication and depends on a multiprotein complex called cohesin. Attachment of sister kinetochores to the mitotic spindle during mitosis generates forces that would immediately split sister chromatids were it not opposed by cohesion. Cohesion is essential for the alignment of chromosomes in metaphase but must be abolished for sister separation to start during anaphase. In the budding yeast *Saccharomyces cerevisiae*, loss of sister-chromatid cohesion depends on a separating protein (separin) called Esp1 and is accompanied by dissociation from the chromosomes of the cohesion subunit Scc1. Here we show that Esp1 causes the dissociation of Scc1 from chromosomes by stimulating its cleavage by proteolysis. A mutant Scc1 is described that is resistant to Esp1-dependent cleavage and which blocks both sister-chromatid separation and the dissociation of Scc1 from chromosomes. The evolutionary conservation of separins indicates that the proteolytic cleavage of cohesion proteins might be a general mechanism for triggering anaphase.

The separation of sister chromatids at the metaphase-to-anaphase transition is one of the most dramatic events of the eukaryotic cell cycle. As cells enter mitosis, chromosome condensation during prometaphase resolves the bulk of each chromatid's chromatin from that of its sister[1,2]. Chromatids nevertheless remain paired along their entire length during the attachment of chromosomes to the mitotic spindle. Cohesion between sisters resists the pulling forces exerted by microtubles attached to sister kinetochores[3] and thereby ensures that sister chromatids attach to microtubules emanating from opposite spindle poles[4,5]. It has long been suspected that destruction of sister-chromatid cohesion, rather than a major change in traction exerted by the spindle, is responsible for the sudden separation of sister chromatids at the metaphase-to-anaphase transition[3,4]. It is not known what triggers this event in the eukaryotic cell cycle.

There are important clues as to the molecular nature of the cohesive structures that holds sisters together and the mechanism by which it is suddenly broken at the onset of anaphase[6]. In *S. cerevisiae*, cohesion between sister chromatids depends on a multisubunit complex, called cohesin, which contains at least four subunits: Scc1, Scc3, Smc1 and Smc3 (refs 7–9). Cohesion is established during DNA replication with the help of Scc2 and Eco1 (refs 9–11). A similar cohesin complex has been implicated in sister-chromatid cohesion in *Xenopus* extracts[2].

In yeast, there is a sudden change in the state of cohesin at the metaphase-to-anaphase transition: two cohesin subunits, Scc1 and Scc3, suddenly disappear from chromosomes at the point when sister chromatids separate[7,9]. The dissociation of Scc1 from chromosomes and the separation of sister chromatids both depend on a 'separin' protein called Esp1 (ref. 12). The existence of Esp1 homologues in many eukaryotes, including humans, suggests that separins have a fundamental and conserved role in chromosome segregation[13–15].

For much of the cell cycle, Esp1 is tightly bound by the anaphase inhibitor Pds1 (ref. 12), whose destruction at the metaphase-to-anaphase transition is triggered by ubiquitination due to the anaphase-promoting complex (APC)[16]. The APC requires an activator protein, Cdc20, to mediate Pds1 destruction[17]. In *S. cerevisiae*, the only role of the APC in promoting sister separation is to destroy Pds1 (refs 12, 18). We now investigate the mechanism by which Esp1 dissolves sister-chromatid cohesion once it has been liberated from Pds1.

Esp1 controls Scc1 chromosome association

The displacement of Scc1 from chromosomes at the metaphase-to-anaphase transition might be a direct effect of Esp1 activity. Alternatively, it might just be a consequence of sister-chromatid separation initiated by Esp1. We therefore examined the mechanism that prevents the association of Scc1 with chromosomes during early G1 phase. Scc1 is destroyed during anaphase and is normally not resynthesized until late G1 in the next cell cycle[7]. However, even when Scc1 is synthesized in early-G1 cells from the galactose-inducible *GAL1-10* promoter, it fails to bind stably to chromosomes[10]. We repeated this experiment using cells arrested in a G1-like state with the mating pheromone α-factor (Fig. 1a). Scc1, induced during pheromone arrest, accumulated in the nuclei of most cells, but bound to chromosomes only weakly, if at all. Furthermore, the protein rapidly disappeared from cells after expression was shut off by addition of glucose (Fig. 1b). When the experiment was repeated with *esp1-1* mutant cells, Scc1 bound to chromosomes with high efficiency and remained associated even after synthesis was terminated (Fig. 1b). This result indicates that Esp1 prevents the stable association of Scc1 with chromosomes during G1 phase, in addition to causing dissociation of Scc1 from chromosomes when sister chromatids separate. Esp1 may therefore have a direct role in removing Scc1 from chromosomes.

An *in vitro* Scc1-dissociation assay

To investigate the mechanism by which Esp1 causes Scc1 to dissociate from chromosomes, we assayed this process *in vitro* (Fig. 2). A crude preparation of yeast chromatin[19], isolated from cells arrested in a metaphase-like state by nocodazole, was incubated with soluble extracts from *esp1-1* mutant cells that either had or had not been induced to overexpress wild-type Esp1 from the *GAL1-10*

promoter. After incubation with both types of extract, the chromatin fraction was again separated from the supernatant by centrifugation and the levels of haemagglutinin(HA)-epitope-tagged Scc1 in chromatin and supernatant fractions were analysed by SDS–PAGE and subsequent immunoblotting. About 70% of the total Scc1 in nocodazole-blocked cells is tightly associated with chromatin[9] and is therefore present in the starting chromatin fraction, which served as substrate. Most Scc1 remained in the chromatin fraction following incubation with extract prepared from *esp1-1* mutant cells, but almost all disappeared from the chromatin fraction after incubation with extract containing overexpressed Esp1 (Fig. 2). Surprisingly, Scc1 induced to dissociate from chromatin by Esp1 appeared in the supernatant fraction as a cleaved product (Fig. 2). Both dissociation of Scc1 from chromatin and its cleavage were inhibited by Pds1 translated in reticulocyte lysate, but not by a control lysate (Fig. 2). We also detected a small amount of Scc1-cleavage activity in extracts prepared from cells not overexpressing Esp1 if they were arrested in G1, and in extracts from cycling cells lacking Pds1 (data not shown). Esp1 also caused ~50% of the Scc3 cohesin subunit[9] to dissociate from chromatin without cleavage. The association of Smc proteins and histone H2B1 with chromatin was unaffected by Esp1-containing extracts (data not shown).

We next investigated the requirements for Scc1 dissociation *in vitro*. It has been suggested that a transient calcium wave in mitotic cells might trigger sister-chromatid separation[20], but Scc1 cleavage was unaltered by addition of the calcium chelator EGTA or by an excess of free calcium. The reaction was also not inhibited by inhibitors of kinases or phosphatases (data not shown). This suggests that the dissociation of Scc1 from metaphase chromatin *in vitro* is neither induced by calcium nor by *de novo* phosphorylation/dephosphorylation. ATP depletion of extracts or addition of the proteasome inhibitor LLNL did not prevent Scc1 cleavage (data not shown), indicating that Scc1 cleavage is probably not due to APC-mediated ubiquitination. We attempted to characterize the proteolytic activity by using protease inhibitors from the four known classes, and found that Scc1 cleavage was only inhibited by high concentrations (10 mM) of *N*-ethylmaleimide.

Scc1 cleaved at anaphase onset *in vivo*

To test whether Esp1-induced cleavage of Scc1 also occurs *in vivo* at the onset of anaphase, we used a yeast strain in which expression of the APC-activator Cdc20 is under the control of the galactose-inducible *GAL1-10* promoter[21]. Cells from this strain were arrested in metaphase by incubation in galactose-free medium and then induced to undergo synchronous anaphase by addition of galactose. Sister-chromatid separation, visualized by the binding to *tet* operators close to CenV of *tet* repressor tagged with green fluorescent protein (GFP) (ref. 7), occurred in most cells within 15 min of Cdc20 induction, and Scc1 dissociated from chromosomes at a similar rate (Fig. 3a, b). We detected a small amount of Scc1 cleavage product of the same size as that seen *in vitro* in cycling cells, but not in cells arrested in metaphase. The cleavage product appeared 15 min after release into anaphase, simultaneously with sister-chromatid separation and the dissociation of Scc1 from chromosomes (Fig. 3c). Full-length Scc1 remaining in cells at this time might originate from the soluble pool of Scc1, whose cleavage is unnecessary for sister separation. Soluble Scc1 in the supernatant fraction of chromatin preparations makes a poor substrate in our *in vitro* cleavage assay (data not shown).

To establish whether cleavage of Scc1 during anaphase depends on Esp1, we compared wild-type and *esp1-1* mutant cells after their release from *GAL–CDC20* arrest at 35 °C. The extent of sister-chromatid separation, Scc1 dissociation from chromosomes (data not shown), and Scc1 cleavage (Fig. 3d) was greatly reduced in the *esp1-1* mutant. We conclude that Esp1 promotes cleavage of Scc1 and its dissociation from chromosomes both *in vivo* and *in vitro*.

A cleavage-resistant Scc1

To determine whether Esp1-mediated cleavage of Scc1 is a cause or a consequence of its dissociation from chromosomes, we first identified the Scc1-cleavage site, with a view to producing a cleavage-resistant mutant. The C-terminal Scc1-cleavage product in anaphase cells (Fig. 3) was immunoprecipitated from cell extracts. Amino-terminal sequence analysis of the fragment showed that cleavage had occurred between a pair of arginine

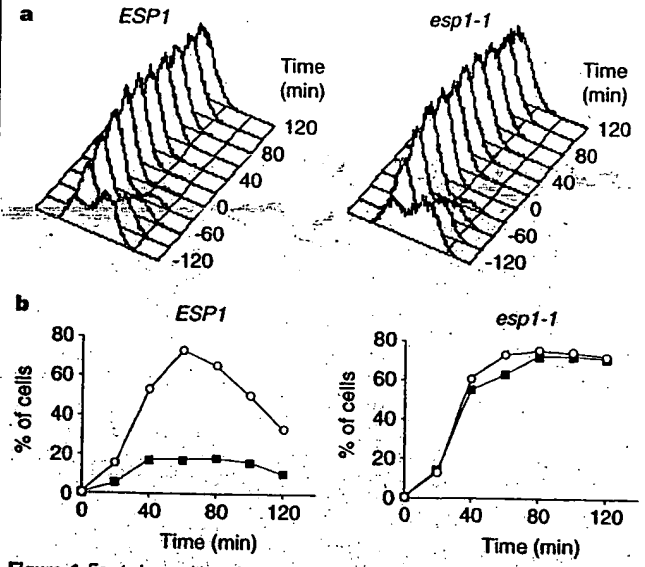


Figure 1 Esp1-dependent chromosome association of Scc1 in G1. a, Strains K7466 (*MATa ESP1 SCC1 GAL-SCC1myc18*) and K7468 (*MATa esp1-1 SCC1 GAL-SCC1myc18*) were arrested in G1 with α-factor for 120 min (time point zero). FACScan analysis showed that cells stayed arrested during the experiment. b, Scc1-Myc18 was induced for 60 min, then cells were transferred to medium containing glucose to repress Scc1-Myc18. Expression of Scc1-Myc18 was seen by whole-cell *in situ* staining (circles), and chromosome binding of Scc1-Myc18 was observed by using chromosome spreads (squares).

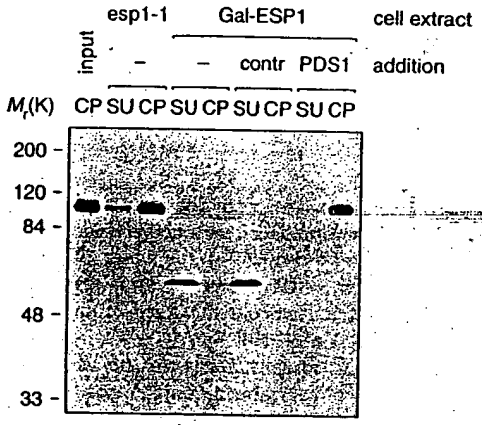


Figure 2 *In vitro* assay for Scc1 dissociation from chromatin. Chromatin was prepared as described[9] from strain K7563 (*MATα SCC1-HA6*) arrested in metaphase by nocodazole treatment. Proteins in the chromatin preparation were resolved by SDS-PAGE; Scc1-HA6 was detected by western blotting (input). This chromatin preparation was resuspended in the indicated extracts, with or without addition of 50% (v/v) *in vitro* translation reactions, as indicated. After incubation, aliquots of the supernatant fraction (SU) and the chromatin fraction (CP) of each reaction were analysed.

residues at positions 268 and 269. The first of these arginine residues was then mutated to aspartic acid (R268D), tagged at the C terminus with HA epitopes, and expressed from the *GAL1-10* promoter: expression of the mutant protein had little effect on cell proliferation. To test whether the R268D mutation had abolished cleavage, we used chromatin from cells expressing it as a substrate in the Esp1 assay. We found that there was no cleavage at site 268, but that the mutant protein was still cleaved in an Esp1-dependent manner (Fig. 4a). The C-terminal cleavage product was now about 10K larger. To identify the second cleavage site, we looked for sequences in Scc1 that are similar to those around the C-terminal cleavage site and found a 5-out-of-7 amino-acid match at position 180 (Fig. 4b). We mutated the arginine before this putative cleavage site to aspartate (R180D).

We next compared the effect of expressing wild-type Scc1, R180D and R268D single-mutant proteins, and the R180D/R268D double-mutant protein from the *GAL1-10* promoter. Neither the single-mutant proteins nor the wild-type protein greatly affected cell proliferation, but expression of the double-mutant protein was lethal (data not shown). We used chromatin from cells transiently expressing the double-mutant protein in our Esp1 assay and found that the R180D/R268D double-mutant protein (Scc1RR-DD) was no longer cleaved. Furthermore, it failed to dissociate from chromosomes (Fig. 4a). The small amount of 'leakage' of Scc1 from chromatin into the supernatant was Esp1-independent (data not shown).

To obtain the N- and C-terminal cleavage products simultaneously, we used as a substrate chromatin from a strain expressing Scc1 tagged N-terminally with a Myc epitope and C-terminally with HA epitope (Fig. 4b). Esp1-mediated cleavage produced a single HA-tagged cleavage fragment but two Myc-tagged fragments, the smaller of which was more abundant (Fig. 4c). These results indicate that all molecules were cleaved at the C-terminal site but not all of them were cleaved at the N-terminal site. A similar pattern of N-terminal cleavage fragments was obtained during anaphase *in vivo* (data not shown).

Non-cleavable Scc1 and sister separation

To investigate why cells expressing the non-cleavable Scc1RR-DD double mutant cannot proliferate, we used centrifugal elutriation to isolate G1 cells from a culture growing without expression of Scc1RR-DD, which were then incubated in the presence and absence of galactose to induce Scc1RR-DD from the *GAL1-10* promoter (Fig. 5). To minimize the duration of mutant protein expression, cells grown in the presence of galactose were transferred to glucose-containing medium after 135 min, when most cells had replicated their DNA (Fig. 5a). In the absence of galactose, sister separation and the dissociation from chromosomes of endogenous Myc-tagged Scc1 occurred simultaneously about 60 min after DNA replication (Fig. 5b–d). Transient expression of Scc1RR-DD, tagged with HA epitope, almost completely prevented sister-chromatid separation (Fig. 5b) but did not affect binding and dissociation of endogenous wild-type Scc1 (Fig. 5c). The mutant protein remained tightly associated with chromosomes long after the endogenous wild-type protein had disappeared (Fig. 5c, d). Scc1RR-DD bound to chromosomes immediately following induction in G1, when wild-type Scc1 is prevented from binding by Esp1 (compare to Fig. 1 and

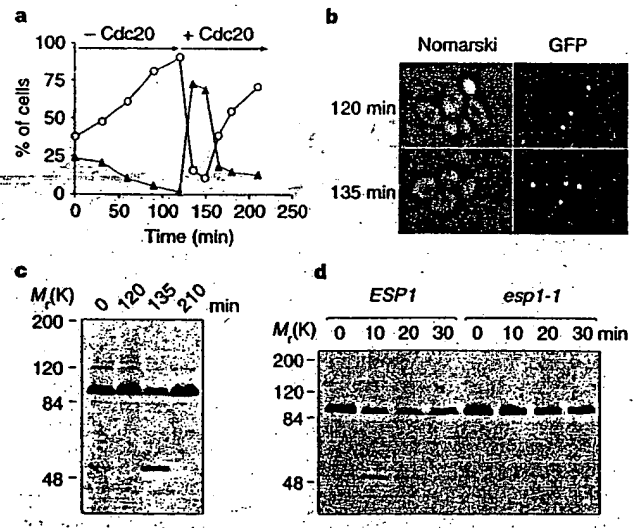


Figure 3 Scc1p cleavage at anaphase onset *in vivo*. **a**, Metaphase arrest and release using Cdc20 depletion of strain K7677 (*MATα cdc20Δ GAL-CDC20 SCC1-HA3 TetR-GFP TetOs*). Scc1-HA3 bound to chromosomes (circles), and the fraction of cells containing two separated GFP dots (triangles). **b**, Examples of cells in the arrest at 120 min, and 15 min after release. **c**, Western blot analysis of Scc1-HA3 in whole-cell extracts prepared from cells at the indicated time points. **d**, As **c**, except that strain K7677 and K8054 (*MATa esp1-1 cdc20Δ GAL-CDC20 SCC1-HA3 TetR-GFP TetOs*) were arrested and released at 35 °C.

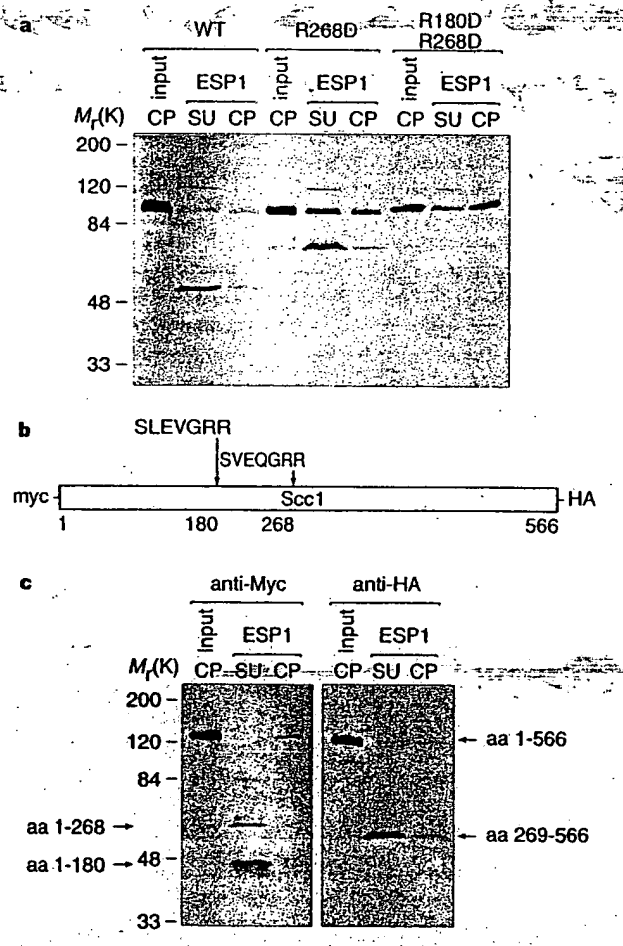


Figure 4 Characterization of the Scc1 cleavage sites. **a**, Strains K8097 (*MATa SCC1-myc18 GAL-SCC1-HA3 TetR-GFP TetOs*), K8099 (*MATa SCC1-myc18 GAL-SCC1(R268D)-HA3 TetR-GFP TetOs*) and K8101 (*MATa SCC1-myc18 GAL-SCC1(R180D, R268D)-HA3 TetR-GFP TetOs*) were grown in medium containing 2% raffinose. Expression of the respective Scc1 variant was induced for 4 h, and cells were arrested by nocodazole treatment. Chromatin was prepared and used in the Esp1 assay. The 120K bands are HA-cross-reacting proteins. WT, wild type. **b**, The cleavage sites in Scc1. **c**, Chromatin was prepared from strain K7768 (*MATα myc9-SCC1-HA6*). Scc1 (ref. 8) and the derived cleavage fragments (aa, amino-acid residues) migrate abnormally slowly during SDS-PAGE, as shown by western blotting with the indicated antibodies.

ref. 10). The failure of Scc1RR-DD to dissociate from chromosomes was not an artefact due to transient overexpression of the protein from the *GAL1-10* promoter, because wild-type Scc1 expressed similarly dissociates from chromosomes with normal kinetics[10].

Expression of the Scc1RR-DD mutant protein caused a transient delay of cytokinesis for 20 min (Fig. 5b), after which cells divided without having separated sister chromatids. Progeny with abnormal DNA contents were produced (Fig. 5a), resembling *esp1-1* mutant cells incubated at the restrictive temperature[22]. The dissociation from chromosomes of wild-type protein on cue shows that Esp1 activity was not impaired in these cells. The failure of sister chromatids to separate even when Esp1 was active also prevented elongation of the mitotic spindle (data not shown), consistent with the idea that loss of cohesion triggers anaphase.

To determine the phenotype of single- and double-mutant Scc1 proteins when expressed from the natural *SCC1* promoter, we transferred the mutations to *SCC1* carried on a centromeric vector. Plasmids carrying the wild-type or either of the single-mutant genes transformed wild-type and *scc1-73* strains and complemented the temperature-sensitive phenotype of the *scc1-73* mutation. No transformants expressing the R180D/R268D double mutant were obtained (data not shown). Together, our results indicate that cleavage of Scc1 at one of two sites is necessary both for sister-chromatid separation and for the dissociation of Scc1 from chromosomes.

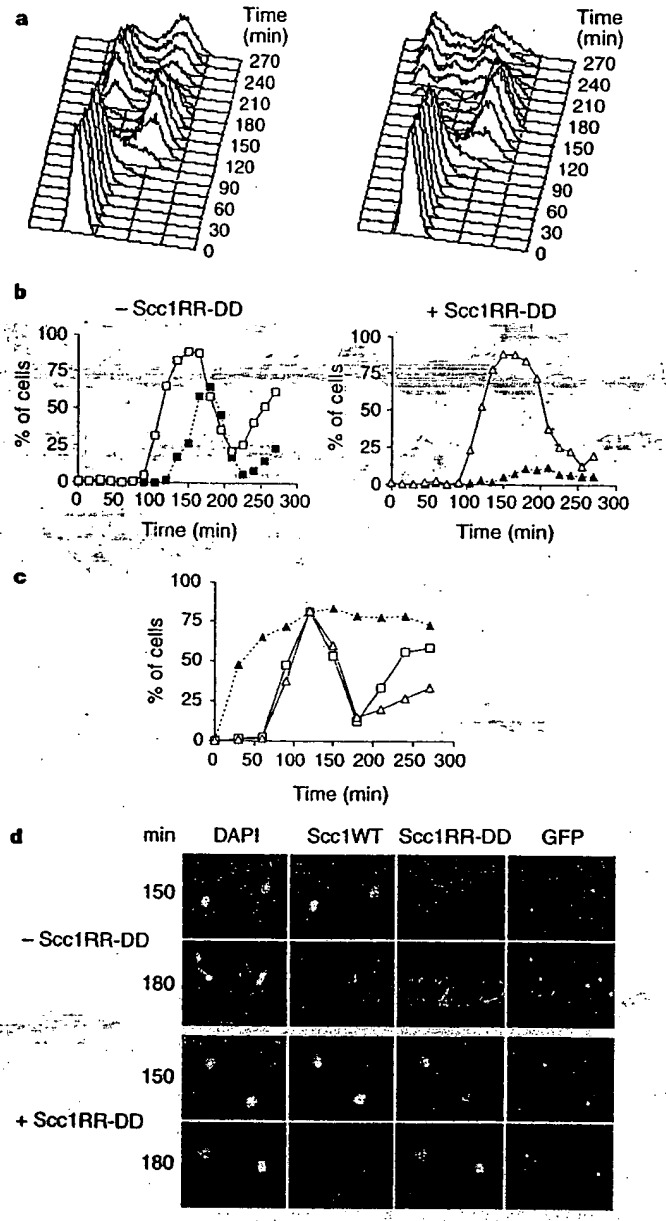


Figure 5 Expression of non-cleavable Scc1 prevents sister chromatid separation. **a**, DNA content as unbudded G1 cells of strain K8101 were released into the cell cycle with or without induction of the Scc1RR-DD mutant. **b**, Budding index (without Scc1RR-DD, open squares; with Scc1RR-DD, open triangles) and percentage of cells with separated sister chromatids (filled symbols). **c**, Scc1 chromosome association. Endogenous wild-type Scc1-Myc18 in cells without (squares) and with Scc1RR-DD expression (open triangles). Scc1RR-DD was tagged with HA epitopes (filled triangles). **d**, Examples of chromosome spreads at 150 min in metaphase and at 180 min when most cells of the control culture had undergone anaphase. DNA was stained with DAPI, Scc1-Myc18 was detected with rabbit anti-Myc antiserum and Cy5-conjugated secondary antibody, Scc1RR-DD-HA3 was detected with mouse monoclonal antibody 16B12 and Cy3-conjugated secondary antibody. Sister chromatids of chromosome V were visible by the GFP dots.

Non-cleavable Scc1RR-DD is functional

To test whether Scc1RR-DD is fully functional apart from its non-cleavability, we first checked whether the double-mutant protein could establish cohesion by itself between sister chromatids in the absence of endogenous Scc1 function, and then whether cohesion established by Scc1RR-DD was dependent on other cohesin subunits. We used centrifugal elutriation to isolate G1 cells of *scc1-73* and *smc3-42* mutant strains[7] that could express Scc1RR-DD from the *GAL1-10* promoter, then incubated them in the presence or absence of Scc1RR-DD at 35 °C, a restrictive temperature for both mutations. In the absence of galactose, sister chromatids separated prematurely in both mutants and failed to segregate to opposite poles of the cell[7] (Fig. 6). Expression of Scc1RR-DD suppressed premature sister-chromatid separation in *scc1-73* mutant cells but not in *smc3-42* cells (Fig. 6). Thus, Scc1RR-DD alone fulfils the cohesion function of Scc1. The cohesion due to Scc1RR-DD depends on Smc3, as does that produced by wild-type Scc1,

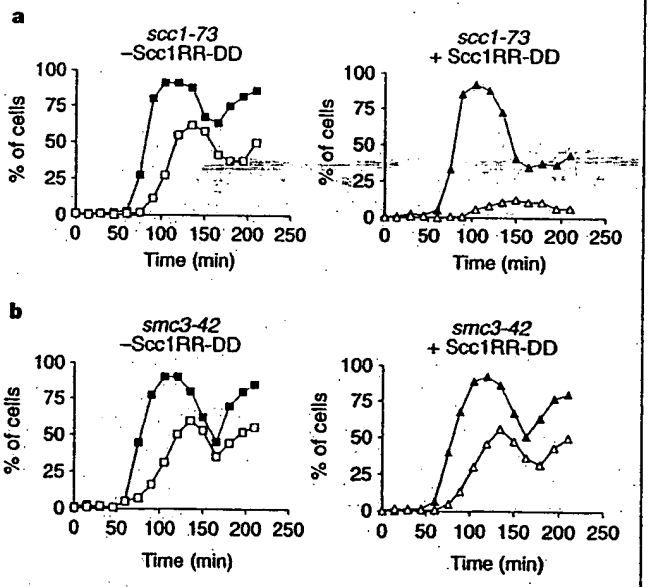


Figure 6 Scc1RR-DD is a functional Scc1 variant. **a**, G1 cells of strain K8103 (*MATa scc1-73 GAL-SCC1(R180D,R268D)-HA3 TetR-GFP TetOs*) were released at 35 °C into medium containing or lacking galactose. The budding index (filled symbols) and the percentage of cells containing separated sister chromatids (open symbols) are shown. **b**, As **a**, except that strain K8149 (*MATa smc3-42 SCC1-myc18 GAL-SCC1(R180D,R268D)*-HA3 TetR-GFP TetOs) was used.

suggesting that Scc1RR-DD differs from wild-type only in its susceptibility to cleavage by Esp1.

Cohesin cleavage triggers anaphase

Our results indicate how cohesion between sister chromatids might be destroyed at the onset of anaphase in yeast (Fig. 7a). As Scc1 is required to maintain cohesion until the metaphase-to-anaphase transition[7–9], its disappearance from chromosomes should destroy cohesion. We have shown that cleavage of Scc1 mediated by the separin Esp1 at the exact point when sister chromatids separate is necessary for sister-chromatid separation, for dissociation of Scc1 from chromosomes, and for the movement of spindle poles to opposite ends of the cell. We propose that sister separation in yeast is triggered by the sudden cleavage of Scc1, although what brings this about is not fully understood. Proteolysis of Pds1 shortly before the metaphase-to-anaphase transition is important: Pds1 inhibits Esp1-dependent cleavage *in vitro* and is destroyed suddenly by APC^{Cdc20} *in vivo* so that sister chromatids can separate[16,17]. The inhibition of APC^{Cdc20} by Mad2 that occurs when chromosomes fail to attach correctly to the mitotic spindle[18,23,24] prevents the destruction of Pds1 and so blocks the onset of anaphase (hence the dubbing of Pds1 and homologues like Cut2 (ref. 25) as securins). Although destruction of Pds1 is necessary for sister separation, it is not sufficient. The disappearance of Scc1 from chromosomes is tightly regulated in yeast cells lacking Pds1 (ref. 26), so a second pathway must exist that regulates either Esp1 activity or the susceptibility of Scc1 to Esp1-dependent cleavage.

Given that Smc1 and Smc3 may form an antiparallel heterodimer[27] and that the link between sisters is a symmetrical, we suggest that Scc1 and Scc3 hold the two Smc1/3 heterodimers together, with each being bound to sister DNA molecules[28] (Fig. 7a); separin could then cleave sister chromatids apart. Cleavage also destabilizes the association of Scc1 with the rest of the cohesin complex. Our results do not indicate whether it is cleavage of Scc1, its subsequent dissociation from the cohesin complex, or both combined that triggers sister-chromatid separation.

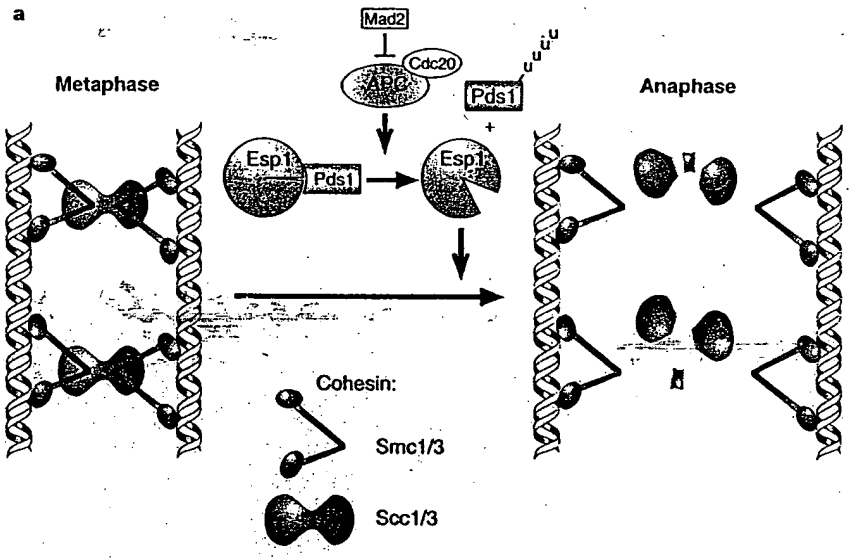
Is c h sin leavage general?

To investigate whether Scc1 was separin's only target, we searched for yeast proteins containing the Scc1 cleavage-site consensus sequences SxExGRR. Only one protein gave a convincing match: we call this protein Rec8 (ref. 29) on the basis of its homology to the *rec8* gene product of fission yeast (Fig. 7b). Rec8 is a member of the Scc1 family and contains two SxExGRK motifs; it is only expressed in meiotic cells and, unlike Scc1, is essential for sister-chromatid cohesion during meiosis I and II (ref. 30; and F. Klein, S. Buonomo and K.N., unpublished results). Rec8 seems to replace Scc1 during meiosis, and cleavage of Rec8 might be crucial for separating sister chromatids during meiosis.

We have not been able to detect Scc1 cleavage motifs in homologous proteins from animals, but the equivalent protein in fission yeast, Rad21 (ref. 31), does contain two near matches in a similar region of the protein to those from Scc1 (Fig. 7b). Most cohesin in animal cells dissociates from chromosomes during prometaphase[2]. A key question for the future is therefore whether the target for animal cell separins is a small fraction of the cohesin pool that persists on metaphase chromosomes or some other cohesion protein.

Is the Esp1 separin a protease?

Although we have not directly determined the identity of the protease that cleaves Scc1, our finding that the cleavage activity in extracts is roughly proportional to their Esp1 concentration (data not shown) raises the possibility that Esp1 is itself the protease. Esp1 and its homologues in other organisms are all large proteins of



b

ScScc1	174	TSLEVGRRF	182
ScScc1	262	NSVEQGRRL	270
ScRec8	425	SSVERGRKR	433
ScRec8	447	RSHEYGRKS	455
SpRad21	173	LSIEAGRNA	181
SpRad21	225	ISIEVGRDA	233
SpRec8	378	SEVEVGRDV	386

Figure 7 Model for separin action on cohesin, and conservation of potential cohesin cleavage sites. **a**, Proteolytic cleavage of one of cohesin's subunits is necessary for sister separation, suggesting that cohesin complexes link sister chromatids. Mad2 is included in the scheme as a known inhibitor of APC^{Cdc20} (ref. 24). **b**, Alignment of known and putative cohesin cleavage sites. Sequence motifs similar to the consensus between the two Scc1 cleavage sites were searched for using the HMMER algorithm[36].

relative molecular mass ~200K (refs 13, 15, 22, 32). Most of their sequences are not highly conserved, but they all contain a conserved C-terminal domain, which might be responsible for a proteolytic activity. This 'separin domain' does not resemble any known protease. However, the insensitivity of the cleavage reaction to many known protease inhibitors suggests that the protease may have a novel mechanism of action. If Esp1 is not itself the protease, then it might instead be an allosteric effector of a protease, which either resides on chromatin or is present in the soluble fraction. Indeed, Esp1 might have functions in addition to Scc1 cleavage[37]. It will be necessary to purify Esp1 and provide it with a more clearly defined substrate to answer these questions. □

Methods

Plasmids and strains. The Scc1 coding sequence was cloned under control of the *GAL1-10* promoter into a YIplac128 derived vector[33]. A DNA fragment encoding 3 tandem HA epitopes was inserted into a *Not*I restriction site introduced by PCR at the C terminus of *SCC1*. Site-directed mutagenesis was performed by exchanging restriction fragments from Scc1 with PCR fragments obtained using primers containing the desired nucleotide changes.

All strains used were derivatives of W303. Epitope tags at the C terminus of the endogenous Scc1p were generated by a PCR one-step tagging method (W. Zachariae and K.N., unpublished results). The Myc-epitope tag at the N terminus of endogenous Scc1 was obtained by integration of a N-terminally tagged portion of Scc1 at the *SCC1* locus. Strains expressing Scc1-Myc18, Esp1, and Cdc20 under control of the *GAL1-10* promoter have been described[7,12,21].

Cell-growth and cell-cycle experiments. Cells were grown in complete medium[34] at 25 °C unless otherwise stated. Strains expressing Cdc20, Esp1 or Scc1 from the *GAL1-10* promoter were grown in complete medium containing 2% raffinose as carbon source. The *GAL1-10* promoter was induced by adding 2% galactose. A G1-like arrest was achieved by adding 1 μg ml^{-1} of the pheromone α-factor to the medium. For metaphase arrest, 15 μg ml^{-1} nocodazole was added with 1% DMSO. Metaphase arrest due to Cdc20 depletion was obtained in cells with Cdc20 under control of the *GAL1-10* promoter by shifting to medium containing raffinose only. For release from the arrest, 2% galactose was added back to the culture.

***In vitro* assay for Esp1 activity.** A crude Triton X-100-insoluble chromatin preparation was obtained from yeast cells as described[19]. The pelleted chromatin was resuspended in yeast cell extracts that had been prepared similarly to the supernatant fraction of the chromatin preparation. Routinely, one tenth volume of an ATP regenerating system (50 mM HEPES/KOH, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 600 mM creatine phosphate, 1.5 mg ml^{-1} phophocreatine kinase, 1 mM DTT, 10% glycerol) was added. (This addition was later found not to be essential.) Reactions were incubated for 10 min at 25 °C with gentle shaking, and stopped on ice. The chromatin fraction was separated again from the supernatant by centrifugation, and resuspended in buffer EBX[19]. Equivalent aliquots of supernatant and chromatin pellet were analysed by SDS–PAGE and western blotting. HA-tagged Scc1 was detected using monoclonal antibody 16B12, Myc-tagged Scc1 with monoclonal antibody 9E10. As overexpression of Esp1 from the *GAL1-10* promoter is toxic to cells, extracts with overproduced Esp1 were prepared 2 h after induction with galactose of a culture pregrown in medium containing raffinose only.

Protein sequencing of the Scc1 cleavage site. The C-terminal Scc1 cleavage fragment was isolated from strain K7756 (*MATa cdc20Δ GAL-CDC20 SCC1-myc18*). Cells synchronized in anaphase were obtained as described for Fig. 3. Protein extract of 5 × 10^9 cells was prepared by breakage with glass beads in breakage buffer (50 mM HEPES/KOH, pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 0.25% Triton X-100, 0.1% SDS, plus protease inhibitors). Myc-epitope-tagged protein was immunoprecipitated with 20 μg anti-Myc 9E11 monoclonal antibody, resolved on SDS–PAGE and transferred to a PVDF membrane[35]. N-terminal sequencing of the band corresponding to the Scc1 cleavage fragment was performed using an Applied Biosystems 492A sequencer. The amino-acid sequence was RLGESIM, corresponding to the Scc1 amino-acid residues from position 269.

Chromosome spreading. Analysis of DNA content and chromosome spreading have been described[7], but spheroplastation to prepare cells for spreading was at 37 °C for only 5 min.

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1. Gimenez-Abian, J. F., Clarke, D. J., Mullinger, A. M., Downes, C. S. & Johnson, R. T. A postprophase topoisomerase II-dependent chromatid core separation step in the formation of metaphase chromosomes. *J. Cell. Biol.* 131, 7–17 (1995).
2. Losada, A., Hirano, M. & Hirano, T. Identification of *Xenopus* SMC protein complexes required for sister chromatid cohesion. *Genes Dev.* 12, 1003–1012 (1998).
3. Nicklas, R. B. The forces that move chromosomes in mitosis. *Annu. Rev. Biophys. Chem.* 17, 431–449 (1988).
4. Miyazaki, W. Y. & Orr-Weaver, T. L. Sister chromatid cohesion in mitosis and meiosis. *Annu. Rev. Genet.* 28, 167–187 (1994).
5. Rieder, C. L. & Salmon, E. D. The vertebrate cell kinetochore and its roles during mitosis. *Trends Cell Biol.* 8, 310–318 (1998).
6. Nasmyth, K. Separating sister chromatids. *Trends Biochem. Sci.* 24, 98–104 (1999).
7. Michaelis, C., Ciosk, R. & Nasmyth, K. Cohesins: Chromosomal proteins that prevent premature separation of sister chromatids. *Cell* 91, 35–45 (1997).
8. Guacci, V., Koshland, D. & Strunnikov, A. A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of *MCD1* in *S. cerevisiae*. *Cell* 91, 47–57 (1997).
9. Toth, A. *et al.* Yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. *Genes Dev.* 13, 320–333 (1999).
10. Uhlmann, F. & Nasmyth, K. Cohesion between sister chromatids must be established during DNA replication. *Curr. Biol.* 8, 1095–1101 (1998).
11. Skibbens, R. V., Corson, L. B., Koshland, D. & Hieter, P. Ctf7p is essential for sister chromatid cohesion and links mitotic chromosome structure to the DNA replication machinery. *Genes Dev.* 13, 307–319 (1999).
12. Ciosk, R. *et al.* An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell* 93, 1067–1076 (1998).
13. May, G. S., McGoldrick, C. A., Holt, C. L. & Denison, S. H. The *bimB3* mutation of *Aspergillus nidulans* uncouples DNA replication from the completion of mitosis. *J. Biol. Chem.* 267, 15737–15743 (1992).
14. Funabiki, H., Kumada, K. & Yanagida, M. Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. *EMBO J.* 15, 6617–6628 (1996).
15. Nagase, T., Seki, N., Ishikawa, K., Tanaka, A. & Nomura, N. Prediction of the coding sequences of unidentified human genes. V. The coding sequences of 40 new genes (KIAA0161–KIAA0200) deduced by analysis of cDNA clones from human cell line KG-1. *DNA Res.* 3, 17–24 (1996).
16. Cohen-Fix, O., Peters, J.-M., Kirschner, M. W. & Koshland, D. Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev.* 10, 3081–3093 (1996).
17. Peters, J.-M. SCF and APC the Yin and Yan of cell cycle regulated proteolysis. *Curr. Opin. Cell Biol.* 10, 759–768 (1998).
18. Yamamoto, A., Guacci, V. & Koshland, D. Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s). *J. Cell Biol.* 133, 99–110 (1996).
19. Liang, C. & Stillman, B. Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. *Genes Dev.* 11, 3375–3386 (1997).
20. Groigno, L. & Whitaker, M. An anaphase calcium signal controls chromosome disjunction in urchin embryos. *Cell* 92, 193–204 (1998).
21. Lim, H. H., Goh, P.-Y. & Surana, U. Cdc20 is essential for the cyclosome-mediated proteolysis of both Pds1 and Clb2 during M phase in budding yeast. *Curr. Biol.* 8, 231–234 (1998).
22. McGrew, J. T., Goetsch, L., Byers, B. & Baum, P. Requirement for ESP1 in the nuclear division of *S. cerevisiae*. *Mol. Biol. Cell* 3, 1443–1454 (1992).
23. Rudner, A. D. & Murray, A. W. The spindle assembly checkpoint. *Curr. Opin. Cell Biol.* 8, 773–780 (1996).
24. Amon, A. The spindle checkpoint. *Curr. Opin. Gen. Dev.* 9, 69–75 (1999).
25. Funabiki, H. *et al.* Cut2 proteolysis required for sister-chromatid separation in fission yeast. *Nature* 381, 438–441 (1996).
26. Alexandru, G., Zachariae, W., Schleiffer, A. & Nasmyth, K. Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. *EMBO J.* 18, 2707–2721 (1999).
27. Melby, T. E., Ciampaglio, C. N., Briscoe, G. & Erickson, H. P. The symmetrical structure of structural maintenance of chromosomes (SMC) and MukB proteins: long, antiparallel coiled coils, folded at a flexible hinge. *J. Cell Biol.* 142, 1595–1604 (1998).
28. Akhmedov, A. T. *et al.* Structural maintenance of chromosomes protein C-terminal domains bind preferentially to DNA with secondary structure. *J. Biol. Chem.* 273, 24088–24093 (1998).
29. Lin, Y., Larson, K. L., Dorer, R. & Smith, G. R. Meiotically induced rec7 and rec8 genes of *Schizosaccharomyces pombe*. *Genetics* 132, 75–85 (1992).
30. Molnar, M., Bahler, J., Sipicki, M. & Kohli, J. The *rec8* gene of *Schizosaccharomyces pombe* is involved in linear element formation, chromosome pairing and sister-chromatid cohesion during meiosis. *Genetics* 141, 61–73 (1995).
31. Birkenbihl, R. P. & Subramani, S. The *rad21* gene product of *Schizosaccharomyces pombe* is a nuclear, cell cycle-regulated phosphoprotein. *J. Biol. Chem.* 270, 7703–7711 (1995).
32. Uzawa, S., Samejima, I., Hirano, T., Tanaka, K. & Yanagida, M. The fission yeast cut1+ gene regulates spindle pole duplication and has homology to the budding yeast ESP1 gene. *Cell* 62, 913–925 (1990).
33. Gietz, R. D. & Sugino, A. New yeast–*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six base pair restriction sites. *Gene* 74, 527–534 (1988).
34. Rose, M. D., Winston, F. & Hieter, P. *Laboratory Course Manual for Methods in Yeast Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1990).
35. Eckerskorn, C. & Lottspeich, F. Structural characterization of blotting membranes and the influence of membrane parameters for electroblotting and subsequent amino acid sequence analysis of proteins. *Electrophoresis* 14, 831–838 (1993).
36. Eddy, S. R. Hidden Markov models. *Curr. Opin. Struct. Biol.* 6, 361–365 (1996).
37. Kumada, K. *et al.* Cut1 is loaded onto the spindle by binding to Cut2 and promotes anaphase spindle movement upon Cut2 proteolysis. *Curr. Biol.* 8, 633–641 (1998).

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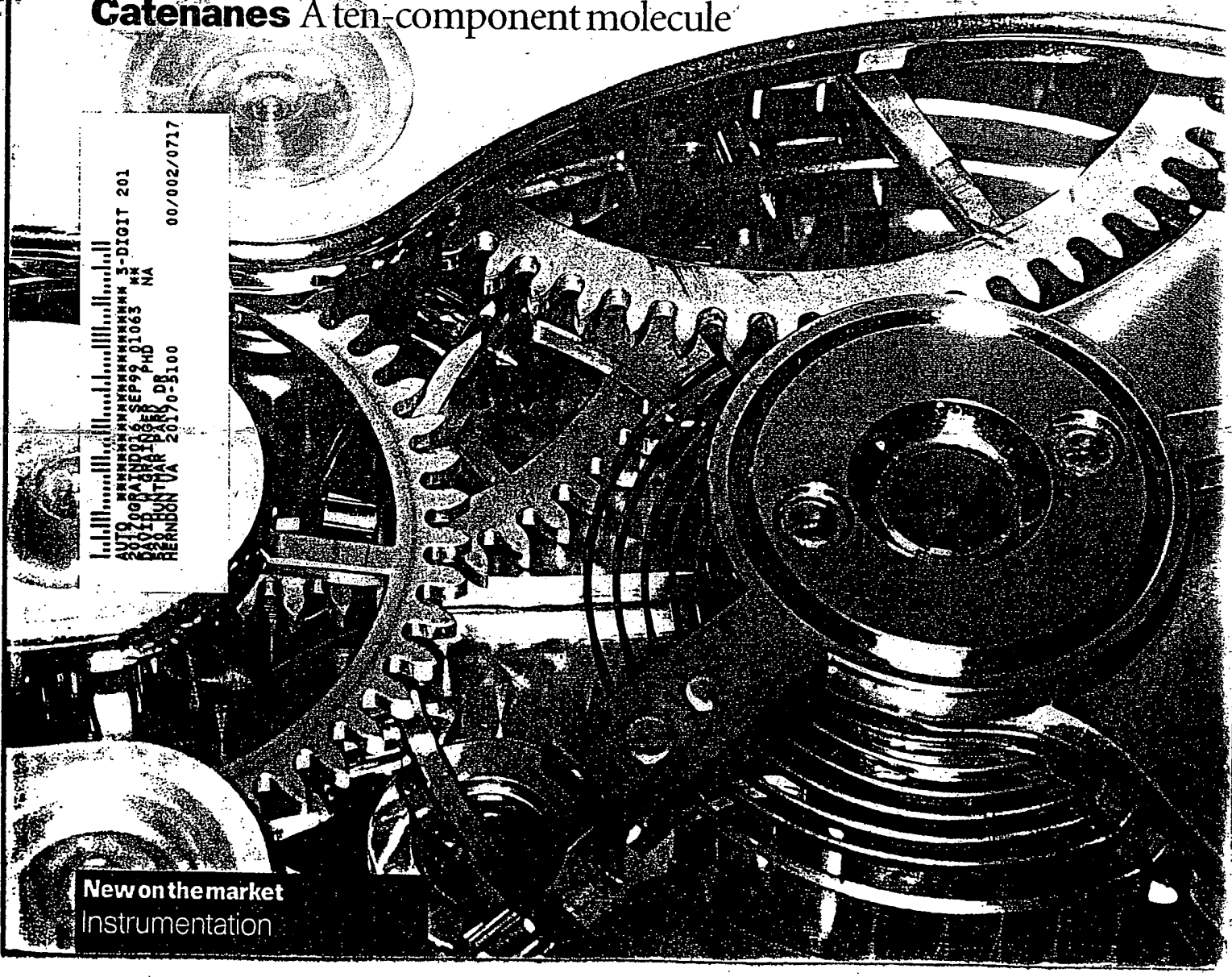

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Two Distinct Pathways Remove Mammalian Cohesin from Chromosome Arms in Prophase and from Centromeres in Anaphase

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Summary

In yeast, anaphase depends on cohesin cleavage. How anaphase is controlled in vertebrates is unknown because their cohesins dissociate from chromosomes before anaphase. We show that residual amounts of the cohesin SCC1 remain associated with human centromeres until the onset of anaphase when a similarly small amount of SCC1 is cleaved. In *Xenopus* extracts, SCC1 cleavage depends on the anaphase-promoting complex and separin. Separin immunoprecipitates are sufficient to cleave SCC1, indicating that separin is associated with a protease activity. Separin activation coincides with securin destruction and partial separin cleavage, suggesting that several mechanisms regulate separin activity. We propose that in vertebrates, a cleavage-independent pathway removes cohesin from chromosome arms during prophase, whereas a separin-dependent pathway cleaves centromeric cohesin at the metaphase–anaphase transition.

Introduction

In eukaryotes, replicated DNA molecules remain associated from the time of their synthesis until they are separated in anaphase. This cohesion between sister chromatids allows the bipolar attachment of chromosomes to the mitotic spindle long after replication has occurred. Loss of cohesion is therefore required for the separation of sister chromatids. In presumably all eukaryotes this event is initiated by activation of the anaphase-promoting complex (APC), a ubiquitin-protein ligase that targets mitotic proteins for destruction by the 26S proteasome (reviewed by Peters, 1999).

How activation of the APC initiates sister separation is best understood in budding yeast (reviewed by Dej and Orr-Weaver, 2000; Koshland and Guacci, 2000; Nasmyth et al., 2000). In this organism, protein complexes known as 14S cohesin are essential for sister chromatid cohesion. Yeast mutants defective in the cohesin subunit genes smc1, smc3, scc1/mcd1, or scc3 separate sister chromatids in the absence of APC activity (Guacci et al., 1997; Michaelis et al., 1997; Toth et al., 1999). This result and the observation that cohesin subunits dissociate from chromatin at the onset of anaphase suggest that the APC initiates anaphase by removing cohesins from chromosomes. The APC mediates this event by ubiquitinating Pds1p (Cohen-Fix et al., 1996), a protein that binds and presumably inhibits the anaphase activator Esp1p (Ciosk et al., 1998). Following ubiquitin-dependent proteolysis of Pds1p, Esp1p mediates the dissociation of 14S cohesin from chromosomes. This reaction depends on cleavage of cohesin's subunit Scc1p/Mcd1p, suggesting an elegant model for the initiation of anaphase (Uhlmann et al., 1999). According to this model, cohesin itself would physically connect sisters until Scc1p/Mcd1p is cleaved in an Esp1p-dependent manner, thereby liberating sisters for poleward movement in anaphase. To illustrate its activating role in the separation of sister chromatids, Esp1p and its orthologs in other eukaryotes are now called separins, whereas Pds1p and its orthologs are called securins (Yanagida, 2000).

Several observations suggest that ubiquitin-dependent proteolysis mediated by the APC and its mitotic activator CDC20/Fizzy is essential for anaphase, not only in budding yeast, but also in other eukaryotes (reviewed in Peters, 1999). For example, APC-dependent proteolysis of the securins Cut2p and PTTG is required for anaphase in fission yeast and *Xenopus*, respectively (Holloway et al., 1993; Funabiki et al., 1996a; Zou et al., 1999). In *Drosophila*, degradation of Pimples is required for anaphase, suggesting that this protein functions as a securin in flies (Stratmann and Lehner, 1996; Leismann et al., 2000). Surprisingly, *cut2* and *pimples* mutants are unable to enter anaphase, suggesting that securins have activating as well as inhibitory roles in sister separation. The function of separins may also be conserved because the separins Cut1p and BIMB are essential for anaphase in fission yeast and Aspergillus, respectively (May et al., 1992; Funabiki et al., 1996b).

The notion that the APC-separin pathway controls anaphase in all eukaryotes is further supported by the observation that cohesin complexes containing orthologs of Smc1p, Smc3p, Scc1p/Mcd1p, and Scc3p exist in *Xenopus* (Losada et al., 1998) and human cells (Sumara et al., 2000) and by the finding that *Xenopus* 14S cohesin is required for proper sister chromatid cohesion (Losada et al., 1998; E. Vorlaufer and J.-M. P., unpublished data). However, despite these similarities with the yeast complex, vertebrate cohesins have been shown to dissociate from chromosomes already in prophase, i.e., long before sisters separate (Losada et al., 1998; Darwiche et al., 1999; Sumara et al., 2000). Unlike in yeast, this event does not depend on activation of the APC, suggesting that securin destruction and separin activation are not required to remove vertebrate cohesins from condensing chromatin (Sumara et al., 2000). In vertebrates, it is therefore not known how sister chromatid cohesion is maintained between prophase and the onset of anaphase, and it is unclear how the APC-separin pathway controls anaphase in eukaryotes other than budding yeast.

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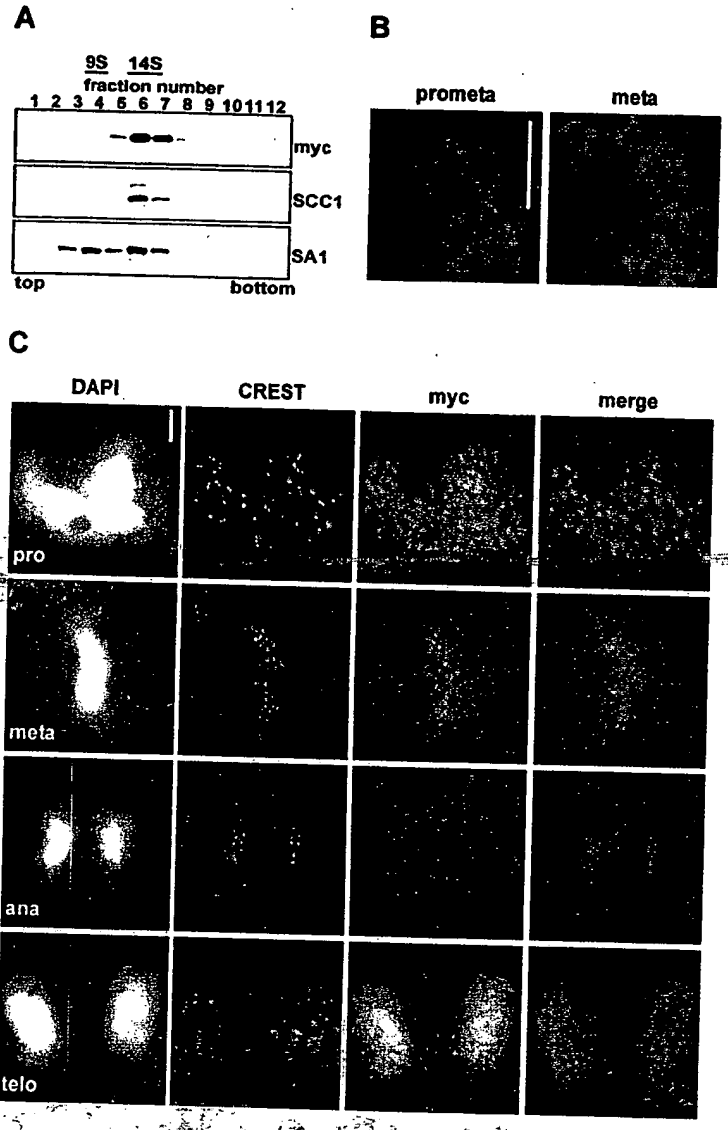

Figure 1. Localization of SCC1 on Metaphase Chromosomes

(A) Sucrose density gradient fractionation of an extract from logarithmically growing HeLa SCC1-myc cells. The proteins indicated were detected with specific antibodies. The upper and the lower band in the middle panel represent myc-tagged and endogenous SCC1, respectively. SA1 is a subunit of human cohesin complexes (Sumara et al., 2000). The positions corresponding to sedimentation coefficients of 9S and 14S are indicated.

(B and C) Localization of SCC1-myc in extracted cells at different stages of mitosis. Exponentially growing HeLa cells expressing SCC1-myc were extracted, fixed, and stained with an anti-myc antibody revealed by an Alexa 568–coupled secondary antibody. Kinetochores were visualized by staining with human CREST serum followed by an Alexa 488–coupled secondary antibody. DNA was counterstained with DAPI. In the merged panels anti-myc staining is in red and CREST staining is depicted in green. SCC1-myc staining is present in extracted SCC1-myc HeLa cells on prophase and telophase chromatin. Some SCC1-myc staining remains on chromosomes in metaphase and disappears from chromatin in anaphase. Further magnification reveals that the remaining staining on metaphase chromosomes is predominantely centromeric, whereas in prometaphase, chromosome arms and centromeres are stained (B). Bars, 5 μm.

To address these questions, we have reinvestigated the subcellular distribution of the cohesin subunit SCC1 in human mitotic cells. We show that a small amount of SCC1 remains associated with metaphase chromosomes where it preferentially localizes to centromeres until it disappears in anaphase. A similarly small amount of SCC1 is cleaved in vivo specifically in anaphase, but not in pro- or metaphase. Reconstitution of SCC1 cleavage in *Xenopus* egg extracts shows that this reaction depends on the APC and separin and affects preferentially chromosome-associated SCC1. Immunopurified separin is sufficient to cleave the SCC1 subunit of purified cohesin complexes, indicating that separin is associated with a protease activity. Mitotic activation of separin depends on the APC and correlates with securin destruction and with partial cleavage of separin itself, suggesting that separin activity is controlled by at least two mechanisms. Based on our results, we propose that vertebrate cohesins are regulated by two different pathways that affect cohesins in a spatially and temporally distinct manner: in prophase, a cleavage-independent pathway removes the bulk of cohesins from the arms of condensing chromosomes, whereas at the metaphase-anaphase transition an APC- and separin-dependent pathway removes centromere-bound cohesin complexes by cleaving their subunit SCC1.

Results

Small Amounts of SCC1 Remain Associated with Centromeric Regions of Human Metaphase Chromosomes

To test if residual amounts of cohesins remain associated with metaphase chromosomes, we reinvestigated the intracellular distribution of SCC1 by immunofluorescence microscopy. Because antibodies to vertebrate cohesins have so far failed to detect their antigens on metaphase chromosomes (Losada et al., 1998; Dar-

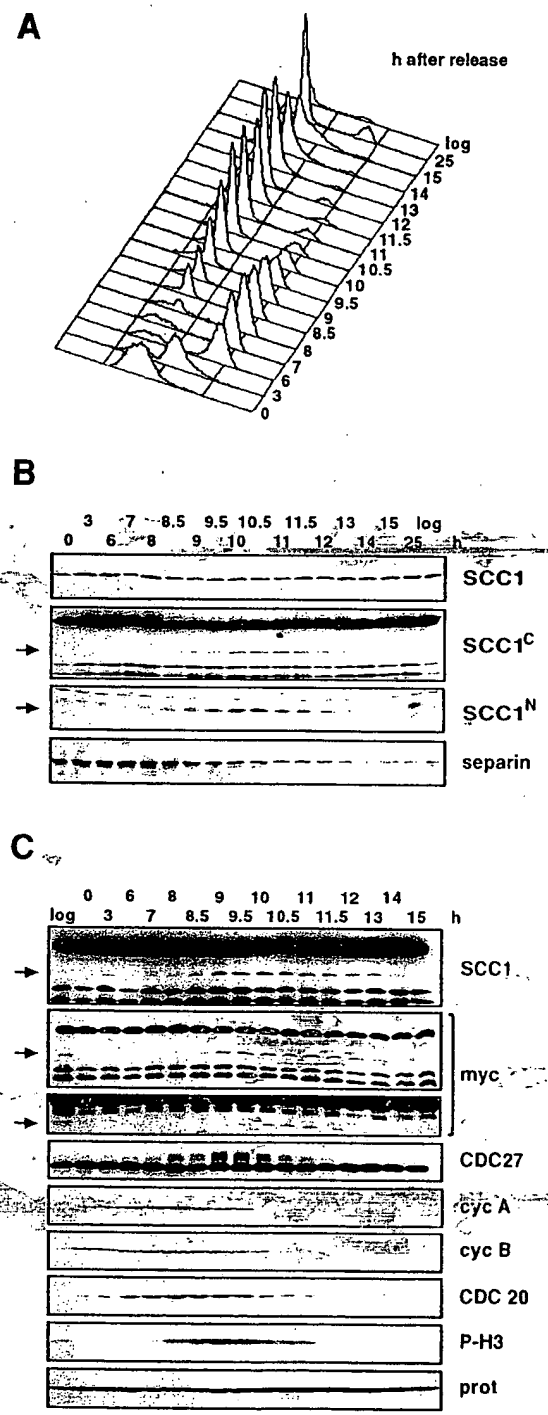

Figure 2. SCC1 Is Cleaved in Mitosis

(A and B) HeLa cells arrested at the onset of S-phase by double-thymidine treatment were released into the cell cycle and samples taken at the indicated time points were analyzed by FACScan (A) and immunoblotting using antibodies against the indicated proteins (B). SCC1, short immunoblot exposure obtained with C-terminal SCC1 antibodies. SCC1^{C}, long exposure of the same SCC1 immunoblot. SCC1^{N}, long immunoblot exposure obtained with N-terminal SCC1 antibodies. Mitosis specific SCC1 cleavage products are marked by arrows. An extract of logarithmically growing HeLa cells (log) was analyzed side by side.

(C) HeLa cells stably expressing SCC1-myc were analyzed by immunoblotting as in (B). Mitosis specific SCC1 cleavage products are marked by arrows. Antibodies to proteasomes (prot) were used as loading controls. cyc A, cyclin A; cyc B, cyclin B; P-H3, histone H3 phosphorylated on serine 10.

wiche et al., 1999; Sumara et al., 2000) we generat d a HeLa cell line stably expressing a tagged version of SCC1 containing 9 myc epitopes at its C terminus. For historic reasons, we used mouse SCC1 (whose amino acid sequence is 94% identical with the human SCC1 sequence) for these experiments. Like endogenous SCC1, ectopically expressed SCC1-myc could be detected in both chromatin and supernatant fractions isolated from logarithmically growing cells, coimmunoprecipitated with other cohesin subunits, was nuclear in interphase cells, and cosedimented with 14S cohesin complexes during sucrose gradient centrifugation (Figure 1A and data not shown). As judged by immunoblotting, SCC1-myc is expressed at levels corresponding to approximately 10% of the endogenous SCC1 (Figure 1A, middle panel), but the ratio of ectopic to endogenous SCC1 may be higher in individual cells because immunofluorescence microscopy revealed that only 80% of all cells expressed detectable amounts of SCC1-myc, and of these, many expressed the ectopic protein at low levels (data not shown).

When we analyzed unextracted mitotic cells expressing SCC1-myc by immunofluorescence microscopy using myc antibodies, only very weak, if any, staining could be seen on metaphase chromosomes (data not shown). Because weak chromosomal signals may be obscured by the strong cytoplasmic signal caused by soluble cohesins, we briefly extracted SCC1-myc expressing cells before fixation and immunostaining. Under these conditions incubation with myc antibodies yielded a fine-punctate staining on metaphase chromosomes of SCC1-myc cells (Figures 1B and 1C). This staining was specific for SCC1-myc because no punctate staining could be observed with myc antibodies on chromosomes of control HeLa cells, or when SCC1-myc cells were stained with antibodies to unrelated proteins or with secondary antibodies alone (data not shown). Coimmunostaining with CREST serum specific for kinetochores (Brenner et al., 1981) revealed that the strongest of the dotty SCC1-myc signals were located precisely in between pairs of kinetochores (Figures 1B and 1C), indicating that SCC1-myc is associated with centromeric regions. In prophase cells, additional SCC1-myc signals could be detected in other chromosomal regions that were not in close proximity to kinetochores (Figure 1C). These noncentromeric signals were often arranged in linear arrays (Figure 1B) and were either largely or entirely absent from metaphase chromosomes (Figures 1B and 1C), suggesting that SCC1 dissociates from chromosome arms between pro- and metaphase but remains associated with centromeres. Importantly, we were unable to detect any clear chromosomal SCC1-myc signals in 140 anaphase cells that we inspected (Figure 1C). Because 80% of all cells in our experiment expressed SCC1-myc, we should have observed some

anaphase cells with chromosomal SCC1-myc signals if they existed. These results suggest that SCC1-myc is removed from centromeric regions at the metaphase to anaphase transition.

A Small Amount of SCC1 Is Cleaved in Human Cells Undergoing Anaphase

To analyze the cell cycle behavior of SCC1 biochemically, HeLa cells were synchronized by a double-thymidine arrest and release protocol. Samples taken at different time points were analyzed for DNA content by FACS (Figure 2A) and for SCC1 by immunoblotting whole cell lysates with antibodies specific for the C terminus of SCC1. Short exposures of chemiluminescent immunoblots showed no fluctuation in SCC1 levels during the cell cycle (Figure 2B, panel SCC1), whereas quantitation of radioactive blots revealed a minor SCC1 decrease of about 15% in mitotic cells (data not shown). Long exposures, however, showed additional bands cross-reacting with SCC1 antibodies (Figure 2B, panel $SCC1^{C}$). Of these, a 95 kDa band appeared specifically as cells progressed through mitosis and disappeared in G1. Antibodies to the N terminus of SCC1 detected a different mitosis-specific band of 30 kDa that appeared and disappeared with similar kinetics as the 95 kDa band (Figure 2B, panel $SCC1^{N}$), consistent with the possibility that a small portion of full-length SCC1 (which migrates as a 125 kDa protein) is cleaved into N- and C-terminal fragments of 30 and 95 kDa, respectively. To verify this hypothesis, we analyzed SCC1-myc in synchronized HeLa cells as above (Figure 2C). Myc immunoblots revealed a mitosis-specific band that migrated as a 120 kDa protein, as expected for a myc-tagged version of the C-terminal SCC1-cleavage fragment (Figure 2C, second panel). In addition, a smaller mitosis-specific fragment of 55 kDa was observed in long exposures of myc immunoblots (Figure 2C, third panel), suggesting that SCC1-myc is cleaved at at least two distinct sites in mitosis.

When we analyzed the behavior of other cell cycle regulators in the same experiments, we observed that the mitosis-specific SCC1 cleavage products began to appear when cyclin A, cyclin B, and CDC20 began to disappear (Figure 2C), i.e., either in meta- or anaphase. To determine more precisely when SCC1 cleavage occurs, we released HeLa SCC1-myc cells from a double thymidine arrest into medium containing nocodazole, a microtubule poison which activates the spindle assembly checkpoint and thereby inhibits the ability of the APC to initiate anaphase (reviewed by Amon, 1999). Under these conditions, cells arrested in a mitotic state with unseparated DNA, highly phosphorylated histone H3 and CDC27 (a subunit of the APC), and high levels of cyclin B and securin, whereas cyclin A was degraded (Figure 3A). Unexpectedly, the levels of CDC20 decreased significantly under these conditions. Longer immunoblot exposures detected small amounts of CDC20, suggesting that these may be sufficient to initiate anaphase upon release from the nocodazole arrest (Figure 3B). Importantly, no mitosis-specific cleavage products of SCC1 could be observed with either SCC1 or myc antibodies in nocodazole-arrested cells (Figure 3A), indicating that SCC1 cleavage depends on the initiation of anaphase and does not occur in pro- or metaphase. This conclusion was confirmed by releasing HeLa SCC1-myc cells from a nocodazole arrest. Under these conditions, SCC1 cleavage occurred when cells began to degrade cyclin B and securin and began to separate their DNA, i.e., in anaphase (Figure 3B).

Xenopus Egg Extracts Recapitulate the Mitosis-Specific Cleavage of Chromatin-Associated SCC1 In Vitro

To study the regulation of SCC1 cleavage, we established an in vitro assay for this reaction. We chose *Xenopus* egg extracts for this purpose because addition of nondegradable cyclin B to such extracts generates a stable mitosis-like state that resembles the situation at the onset of anaphase, i.e., with both cyclin-dependent kinase 1 and APC activities being high (Murray et al., 1989). As a source for SCC1, we used fractions prepared from nocodazole arrested HeLa cells expressing SCC1-myc. When we added low speed pellet fractions enriched in chromatin to mitotic *Xenopus* extracts, the formation of two SCC1-myc cleavage products could be detected by immunoblotting (Figures 4A and 4C). The electrophoretic mobility of these fragments was indistinguishable from the mobility of the cleavage products formed in vivo, indicating that cleavage in vitro occurs at the physiologically relevant sites (Figure 4B). No cleavage could be detected when chromatin from HeLa SCC1-myc cells was incubated in *Xenopus* interphase extracts (Figure 4A), indicating that SCC1 cleavage in vitro is mitosis-specific. When chromatin-free low speed supernatants from HeLa SCC1-myc cells were incubated in mitotic *Xenopus* extracts much less cleavage occurred than in reactions containing chromatin fractions, although roughly equal amounts of SCC1-myc were present in both reactions (Figure 4C). This result was not due to the presence of an inhibitory factor in the supernatant because addition of the supernatant fraction to reactions containing chromatin and mitotic *Xenopus* extract did not inhibit SCC1-myc cleavage (data not shown). These results suggest that chromatin associated SCC1 is preferentially cleaved, whereas soluble SCC1 is a poor substrate.

SCC1 Cleavage Depends on Separin and the APC

Immunoblot experiments showed that both the chromatin pellet and the supernatant fraction of HeLa cell extracts used in this assay contained APC and separin (data not shown). To analyze if these proteins are required for SCC1 cleavage, as they are in budding yeast, we performed a series of immunodepletion experiments. As depletion from the chromatin fraction was technically impossible, we depleted human separin from supernatant fractions of HeLa SCC1-myc cells (Figure 5A). No cleavage of SCC1-myc occurred when these fractions were incubated in mitotic *Xenopus* extract (Figure 5B), suggesting that human separin is required to cleave SCC1-myc. This result also implies that the mitotic *Xenopus* extract alone is not sufficient for cleaving SCC1-myc in this system, raising the possibility that the activity of *Xenopus* separin is labile in the mitotic extracts, or that *Xenopus* separin cannot cleave mammalian SCC1 due to species differences. In contrast, we found that depletion of the APC from HeLa SCC1-myc supernatant

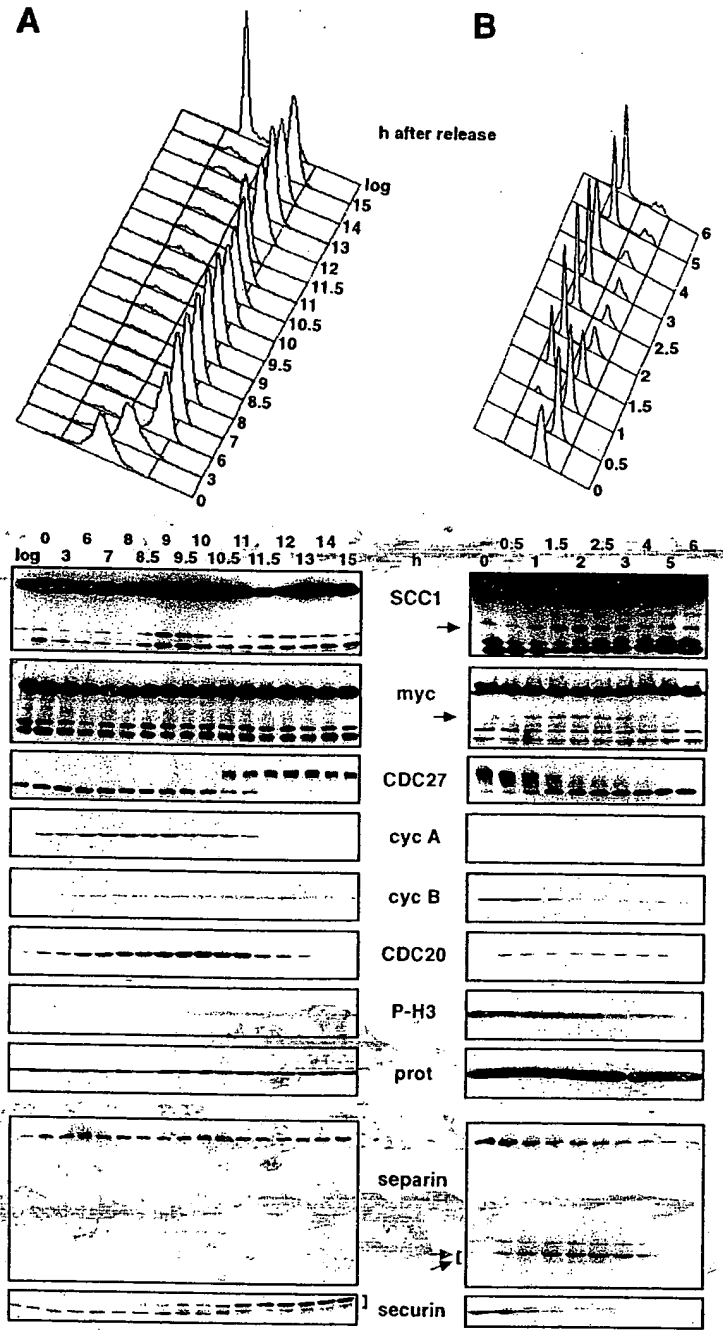

Figure 3. SCC1 Is Cleaved in Anaphase

HeLa SCC1-myc cells were either first synchronized by double-thymidine treatment and subsequently released into the cell cycle in the presence of nocodazole (A) or HeLa SCC1-myc cells were first arrested by nocodazole treatment and then released into the cell cycle (B). Samples were taken at the indicated time points and analyzed by FACScan (top panels) and immunoblotting with antibodies to the indicated proteins (bottom panels). Anaphase-specific cleavage products of SCC1-myc and separin are marked by arrows. The electrophoretic mobility shift of CDC27 and securin is due to mitosis-specific phosphorylation. cyc A, cyclin A; cyc B, cyclin B; P-H3, phosphorylated histone H3; prot, proteasome.

fractions did not abolish SCC1-myc cleavage (data not shown). Also depletion of the APC from mitotic *Xenopus* extracts had little effect (Figures 5A and 5C). Simultaneous depletion of the APC from both HeLa and *Xenopus* extracts did, however, abolish SCC1-myc cleavage (Figure 5C). This result suggests that the APC is required for SCC1-myc cleavage in this assay, and that both human and *Xenopus* APC are sufficient to support this reaction. We presently do not know why mitotic human cell extracts alone are not sufficient to cleave SCC1, but it is possible that mitotic *Xenopus* extracts are required to activate the APC (whose activity is inhibited by MAD2 in nocodazole-arrested human cells) or to mediate efficient proteaseome-dependent proteolysis.

Mitotically Activated Separin Is Sufficient to Cleave Purified Cohesin Complexes

To analyze if separin is directly involved in SCC1 cleavage we immunoprecipitated separin from nocodazole-arrested mitotic HeLa cells (Figures 6A and 6B) and

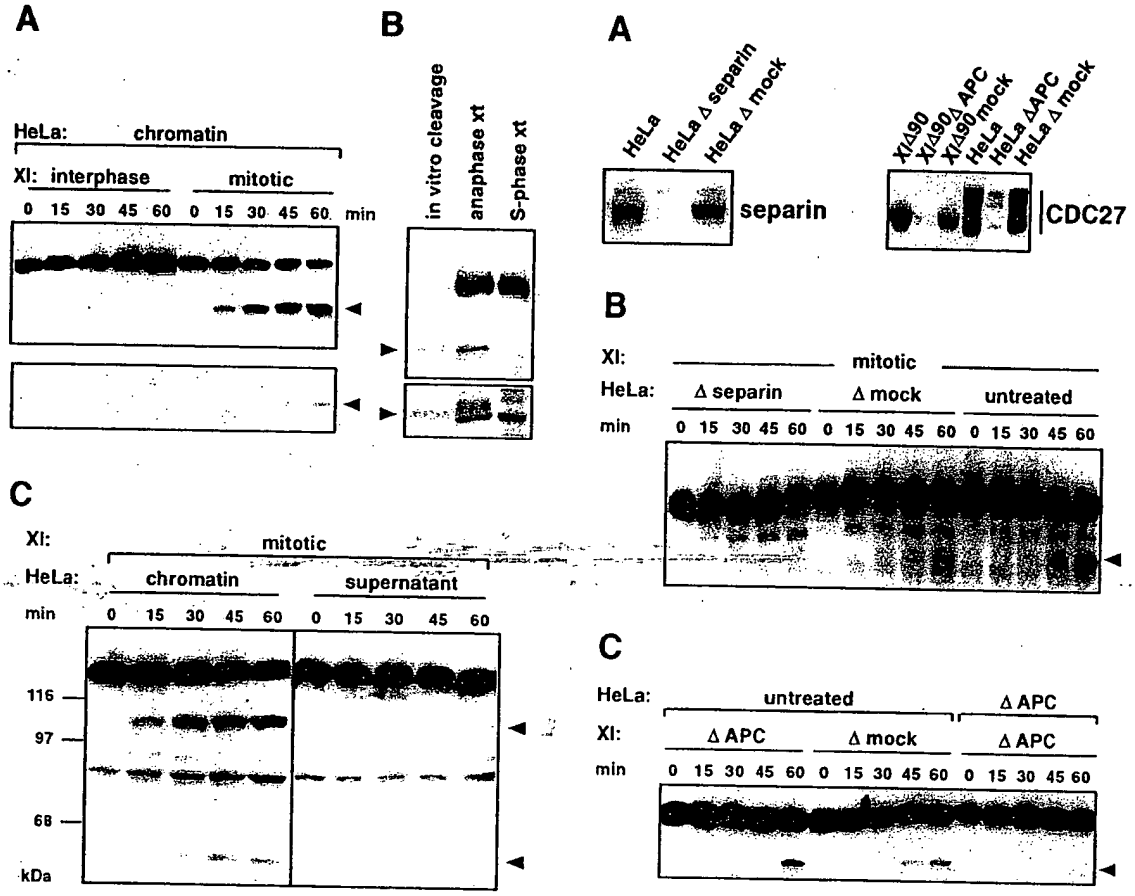

Figure 4. SCC1-myc Is Cleaved in Mitotic *Xenopus* Extracts

(A) Chromatin-enriched fractions from nocodazole-arrested HeLa SCC1-myc extract were incubated either in interphase or mitotic *Xenopus* egg extracts (Xl). Samples were taken at the indicated time points and analyzed by immunoblotting with myc antibodies. Mitosis-specific SCC1-myc cleavage products are marked by arrowheads.

(B) SCC1-myc cleaved in vitro as in (A) and extracts obtained from a double thymidine-release experiment with HeLa SCC1-myc cells (anaphase, S-phase) were analyzed by immunoblotting with myc antibodies. Mitosis-specific SCC1-myc cleavage products are marked by arrowheads.

(C) Chromatin-enriched fractions and chromatin-free supernatant fractions from nocodazole-arrested HeLa SCC1-myc cells were incubated in mitotic *Xenopus* egg extracts (Xl). Samples were taken at the indicated time points and analyzed by immunoblotting with myc antibodies. Mitosis-specific SCC1-myc cleavage products are marked by arrowheads.

Figure 5. Cleavage of SCC1-myc in Mitotic *Xenopus* Extracts Depends on Separin and the APC

(A) Mitotic extracts from *Xenopus* eggs ($Xl^{\Delta 90}$) or from nocodazole-arrested HeLa SCC1-myc cells (HeLa) were either left untreated, depleted with control antibodies (Δmock), or depleted with antibodies to separin (Δseparin) or the APC (ΔAPC) and analyzed by immunoblotting with separin or CDC27 antibodies.

(B) Mitotic *Xenopus* extracts (Xl) were supplemented with the supernatant fractions from untreated, mock- or separin-depleted HeLa SCC1-myc cell extracts obtained as in (A). At the indicated time points, samples were taken and analyzed by immunoblotting with myc antibodies. The slowest migrating band represents full length SCC1-myc. A mitosis-specific SCC1-myc cleavage product is marked by an arrowhead.

(C) APC-depleted or untreated supernatant fractions from HeLa SCC1-myc cells obtained as in (A) were incubated in APC-depleted (Xl: ΔAPC) or mock-depleted (Xl: Δmock) mitotic *Xenopus* extracts and analyzed as in (B).

purified soluble cohesin complexes from supernatant fractions of mitotic HeLa SCC1-myc cells (Figure 6C). When the separin immunoprecipitates washed with buffer were incubated with purified cohesin, no SCC1-myc cleavage could be detected by immunoblotting (Figure 6D, left panel). We therefore incubated the separin immunoprecipitates in mitotic *Xenopus* extracts, speculating that the role of these extracts in the above described assay could be to activate separin. When separin immunoprecipitates were added to mitotic *Xenopus* extracts, reisolated, washed and then incubated with purified cohesin complexes, cleavage of SCC1-myc was observed at the physiologic sites (Figure 6D, right panel). No SCC1-myc cleavage was observed when separin immunoprecipitates were incubated in an interphase *Xenopus* extract (Figure 6D, middle panel), suggesting that separin is activated in the *Xenopus* extract by mitosis-specific mechanisms.

To obtain insight into these mechanisms and to address whether separin itself is the SCC1 protease we analyzed separin immunoprecipitates before and after incubation in mitotic *Xenopus* extracts. Coomassie blue

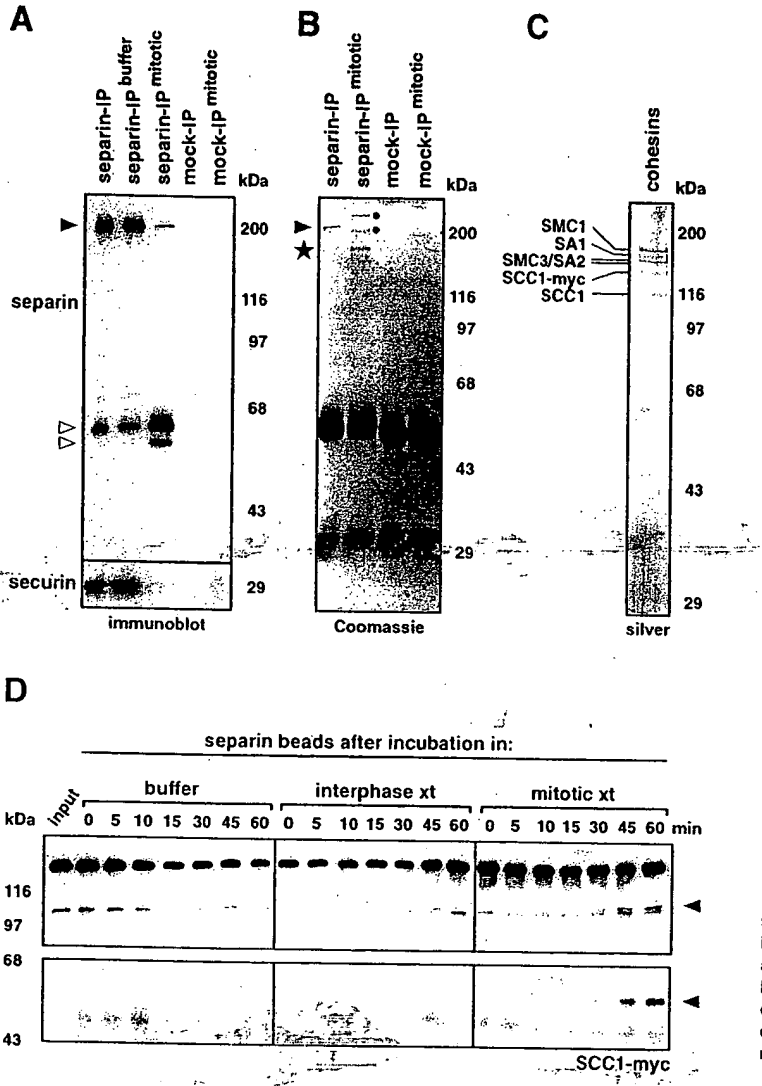

Figure 6. Mitotic Activation Allows Separin Immunoprecipitates to Cleave the SCC1-myc Subunit of Purified Cohesin Complexes and Coincides with Securin Destruction and Separin Cleavage

(A and B) Immunoprecipitates obtained with separin antibodies (separin-IP) or with control antibodies (mock-IP) from extracts of nocodazole-arrested HeLa cells were either left untreated, or incubated in buffer or in mitotic *Xenopus* extracts. Subsequently, the immunoprecipitates were washed and analyzed either by immunoblotting with separin (A, top panel) or securin antibodies (A, bottom panel), or by Coomassie blue staining (B). Full-length separin is marked by a filled arrowhead, a C-terminally truncated form of separin by a star, and mitosis-specific C-terminal separin cleavage products by open arrowheads. Two unidentified *Xenopus* proteins that bind to separin antibody beads in the absence of human separin (see text) are marked by filled dots. The abundant 55 and 29 kDa bands visible in (B) represent IgG heavy and light chains, respectively.

(C) Immunopurified soluble cohesin complexes were analyzed by silver staining. The positions of individual subunits are indicated (see Sumara et al., 2000).

(D) Separin immunoprecipitates were incubated in either buffer, or interphase or mitotic *Xenopus* extracts, washed and added to purified cohesin complexes. Samples were taken at different time points and analyzed by immunoblotting with myc antibodies. The slowest migrating band in the top panels represents full-length SCC1-myc. SCC1-myc cleavage products that are generated in the presence of mitotically activated separin are marked by arrowheads. The bottom panels show a longer exposure of the same blots as in the top panels. Panels (A) and (B) are from a different experiment than panels (C) and (D) but mitotically activated separin immunoprecipitates showed mitosis-specific SCC1-myc cleavage activity in six independent experiments.

staining showed two bands of 200 and 180 kDa in the separin immunoprecipitates before their incubation in *Xenopus* extract which were absent in control precipitates (indicated by a filled arrowhead and a star in Figure 6B). Mass spectrometric analysis of tryptic peptides identified these bands as full length and truncated forms of separin (data not shown). The 200 but not the 180 kDa band could also be recognized by monoclonal antibodies specific for the separin C terminus (Figure 6A), indicating that the 180 kDa separin is truncated at the C terminus. After incubation in mitotic *Xenopus* extract the amount of the 200 kDa separin band was strongly reduced and two additional bands of 220 and 195 kDa were detected by Coomassie staining (Figure 6B, marked by dots). The identity of these 200 and 195 kDa *Xenopus* proteins is presently unknown. However, we do not believe that these proteins are relevant for the SCC1 cleavage reaction because they also bound to separin antibody beads that had not previously been incubated in human extracts, and these immunoprecipitates were unable to cleav SCC1-myc (data not shown).

Immunoblotting with securin antibodies showed that securin was present in the separin immunoprecipitates before but not after incubation in the mitotic *Xenopus* extract (Figure 6A), consistent with the possibility that separin activation in the *Xenopus* extract depends on APC-dependent securin proteolysis. To test this possibility, we incubated separin immunoprecipitates in mitotic *Xenopus* extracts from which the APC had either been immunodepleted (data not shown) or in which APC activity was competitively inhibited by the addition of N-terminal cyclin B peptides (Figure 7A). Under these conditions the separin immunoprecipitates still contained securin after incubation in the *Xenopus* extracts (Figure 7B) and showed a significantly reduced ability to cleave SCC1 (Figure 7C). APC activity is therefore required to activate separin, possibly because securin proteolysis is needed for separin activation.

Immunoblotting with C-terminal separin antibodies confirmed that th majority of full length separin disappeared during incubation in mitotic *Xenopus* xtract (Figure 6A). These blots further revealed that at th same

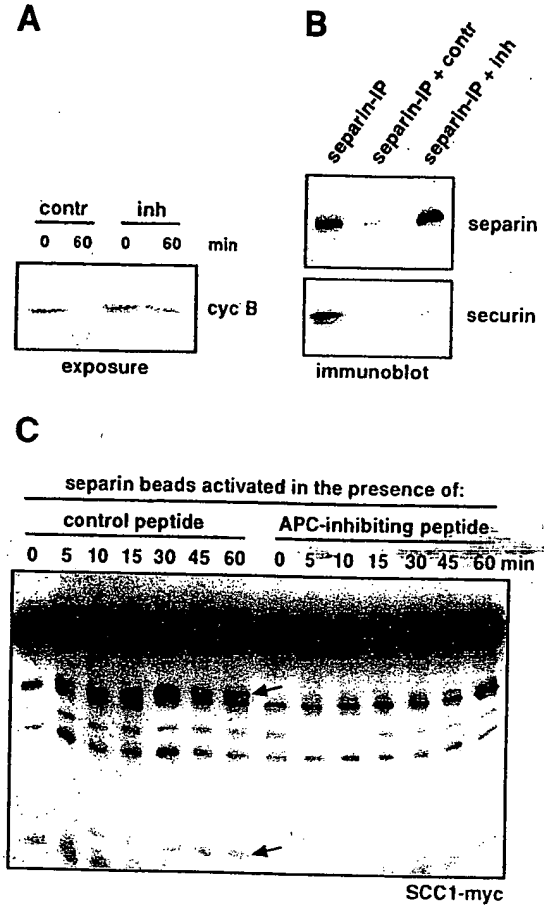

Figure 7. Mitotic Activation of Separin Immunoprecipitates Depends on the APC

(A) In vitro translated ^{35}S-labeled full-length cyclin B was added to mitotic *Xenopus* extracts that were supplemented with 1 mM of N-terminal human cyclin B peptides containing either a mutated (contr) or the wild-type version (inh) of the destruction box and subsequently used for activation of separin immunoprecipitates (see below). Samples were taken at the beginning (0 min) and the end (60 min) of the period during which immunoprecipitates were incubated in the extracts and analyzed by SDS-PAGE and phosphorimaging. Cyclin B proteolysis is blocked by wild-type but not by mutant peptides.

(B) Separin immunoprecipitates (separin-IP) from extracts of nocodazole-arrested HeLa cells were either left untreated or incubated in mitotic *Xenopus* extracts containing peptides as described in (A) and subsequently washed and analyzed by immunoblotting with separin and securin antibodies.

(C) Separin immunoprecipitates obtained as in (B) were incubated with purified cohesin complexes. Samples were taken at different time points and analyzed by immunoblotting with myc antibodies. The slowest migrating band represents SCC1-myc. SCC1-myc cleavage products are marked by arrows.

time a 55 kDa band appeared and a 60 kDa band increased in its abundance, suggesting that separin is partially cleaved in the mitotic *Xenopus* extract (Figure 6A, open arrowheads). The formation of these cleavage products was reduced in *Xenopus* interphase extracts. The activation of separin in mitotic extracts therefore coincides with at least two reactions, proteolysis of separin-associated securin and cleavage of full length separin at at least two distinct sites.

S parin Is Cl aved in Anaphase In Vivo

To address if the cleavage of separin in mitotic *Xenopus* extracts is physiologically relevant, we analyzed th behavior of separin in vivo. Immunoblot experiments revealed that the majority of separin is degraded as HeLa cells progress through mitosis and that separin levels remain low in G1 (Figure 2B). Separin remained stable, however, when cells entered mitosis in the presence of nocodazole, i.e., upon activation of the spindle assembly checkpoint (Figure 3A). When cells exited mitosis after being released from the nocodazole arrest, separin levels began to decrease at the same time as securin and cyclin B degradation occurred (Figure 3B), indicating that separin degradation is initiated in anaphase. The C-terminal monoclonal separin antibodies used in these experiments also detected 60 and 55 kDa bands that specifically appeared when full length separin began to disappear (Figure 3B), demonstrating that separin is also cleaved at at least two sites in anaphase in vivo. The C-terminal separin fragments were not detected in extracts from nocodazole-arrested cells (Figure 3A). The appearance of the separin cleavage products in anaphase and their disappearance in G1 therefore resembles the behavior of SCC1 cleavage products, suggesting that SCC1 and separin cleavage are coregulated and may be mediated by the same pathway.

Discussion

To initiate anaphase, eukaryotic cells have to dissolve the cohesion that holds sister chromatids together. In budding yeast, this event depends on cleavage and subsequent removal of cohesin complexes from chromatin by the APC-separin pathway (Uhlmann et al., 1999). The APC and therefore, possibly also separin are believed to initiate anaphase also in animal cells, but how they do so has remained unclear. This uncertainty stems largely from the observation that in vertebrate cells, cohesins dissociate from chromosomes already in prophase, i.e., long before sisters separate and before the APC-separin pathway is thought to become active (Losada et al., 1998; Darwiche et al., 1999; Sumara et al., 2000). Our results suggest a model that is able to explain this conundrum. In this model, we propose that two distinct pathways regulate the association of cohesins with chromosome arms in prophase and with centromeres at the metaphase-anaphase transition (Figure 8).

A Two-Step Model for the Mitotic Dissociation of Cohesins from Chromosomes

The mechanism by which vertebrate cohesins are removed from chromosomes in prophase is still unknown, but we found that this pathway dissociates cohesins without detectable SCC1 cleavage (Figure 3) and does not require APC activity (Sumara et al., 2000). These observations suggest that separin is not involved in the prophase pathway, explaining why the bulk of cohesins is not degraded during mitosis in vertebrate cells (Figure 2). The solubilization of cohesins at this early stage of mitosis correlates with chromosome condensation and

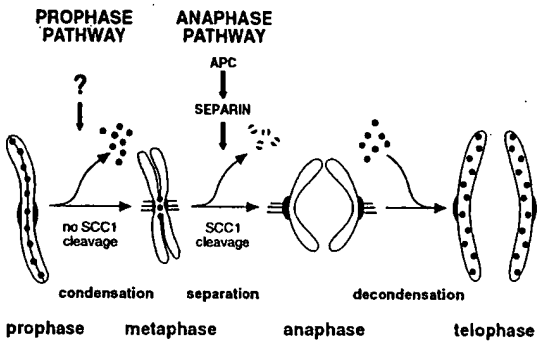

Figure 8. A Model Illustrating How the Association of Vertebrate Cohesin Complexes with Chromosomes Is Regulated in Mitosis

Cohesin complexes are illustrated as black dots and cleaved complexes as split dots.

the visible appearance of discrete sister chromatid arms (Sumner, 1991) and may in fact be required for these processes. This hypothesis could explain why the prophase pathway is largely or entirely absent in budding yeast, where only little mitotic chromosome condensation occurs (Guacci et al., 1994). The prophase pathway removes presumably most, if not all cohesin from chromosome arms but, importantly, it spares SCC1 at centromeric regions (Figure 1), where it may be required to hold sister chromatids together (see below).

Our results show that, at the onset of anaphase, a second pathway is activated which cleaves the SCC1 subunit of cohesin complexes at two distinct sites (Figure 2). This pathway depends on entry into anaphase in vivo (Figure 3) and on the APC and separin in vitro (Figure 5), indicating that it corresponds to the APC-Esp1p pathway that controls the initiation of anaphase in budding yeast (Uhlmann et al., 1999). Several observations suggest that this pathway preferentially cleaves, and thereby solubilizes, cohesin complexes that are bound to chromosomes: First, SCC1 staining disappears from centromeres between metaphase and anaphase (Figure 1), i.e., at the time when SCC1 cleavage is initiated (Figure 2 and 3). Second, in mitotic *Xenopus* extracts, SCC1-myc in chromosome fractions is cleaved more efficiently than soluble SCC1-myc (Figure 4C). Third, most cohesin is not degraded during mitosis in vivo (Figure 2), suggesting that soluble cohesin complexes are not efficiently recognized by the APC-separin pathway. These observations together suggest that APC- and separin-dependent cleavage of SCC1 removes cohesin complexes from centromeres.

Does SCC1 Cleavage Initiate Anaphase?

SCC1 disappears from centromeres when sisters separate (Figure 1) and immunodepletion experiments in *Xenopus* have shown that cohesins are required for proper sister chromatid cohesion not only in yeast but also in vertebrates (Losada et al., 1998; E. Vorlaufer and J.-M. P., unpublished data). The simplest explanation of these observations is that the association of cohesins with centromeres is required to connect sisters until metaphase, and that SCC1 cleavage liberates sisters for poleward movement in anaphase. To test this hypothesis, it will in the future be important to analyze the effects of noncleavable SCC1 mutants and of separin inhibition in vertebrate cells. Despite these open questions, our hypothesis offers a simple explanation for numerous cytologic observations which suggest that arm and centromere cohesion are regulated differently, not only during meiosis, where arm cohesion is lost in anaphase I and centromere cohesion in anaphase II, but also in mitosis (reviewed in Rieder and Cole, 1999). The best known example that supports this hypothesis is the behavior of chromosomes in many plant and animal cells in which the spindle assembly checkpoint has been activated by treatment with microtubule poisons. Under these conditions, many cells arrest in mitosis with chromosomes whose arms have separated but whose centromeres are still connected, yielding the typical X or V shape seen in karyotypic analyses. The spindle assembly checkpoint is believed to cause this state by inhibiting the ability of the APC to initiate anaphase (reviewed by Amon, 1999). Similar chromosomal configurations are seen when the anaphase regulators CDC20/Fizzy, Pimples, or Three-rows are mutated in *Drosophila* (Sigrist et al., 1995; Stratmann and Lehner, 1996). Our results suggest that under all these conditions, centromeric cohesion is maintained because SCC1 cannot be cleaved and because cohesin complexes can therefore not be removed from centromeres. In contrast, the prophase pathway that solubilizes cohesin complexes without SCC1 cleavage would be activated normally under these conditions, explaining why arm cohesion is lost.

Regulation of Arm versus Centromere Cohesion

Our observation that SCC1 is regulated differently at chromosome arms and centromeres raises important questions about how the prophase and the anaphase pathway are able to distinguish between cohesin complexes at these two locations. We have recently discovered that human cells contain two distinct cohesin complexes differing slightly in their subunit composition, but cytologic experiments suggest that the bulk of both of these complexes dissociates from chromatin in prophase (Sumara et al., 2000). There is therefore presently no indication that cohesin complexes located on arms and at centromeres differ in their subunit composition. Instead, specialized centromeric proteins may protect centromeric cohesin complexes from solubilization by the prophase pathway. For example, the *Drosophila* protein MEI-S332 has been discussed as a candidate for a protein that protects centromeric cohesion (Tang et al., 1998; Rieder and Cole, 1999).

There also has to exist a mechanism that restricts SCC1 cleavage at the onset of anaphase to cohesin complexes on chromosomes, leaving SCC1 in the bulk of soluble complexes uncleaved. This is not achieved by restricting the localization of separin to chromosomes because biochemical and cytologic experiments indicate that human separin is largely soluble (I. C. W. and J.-M. P., unpublished data). However, other coactivators of SCC1 cleavage could be specifically present at centromeres. For example, several protein kinases have been localized to kinetochore/centromere regions (reviewed by Pidoux and Allshire, 2000), raising the possibility that such enzymes may help to render centromeric

SCC1 cleavage competent. Alternatively, it is possible that the solubilization of cohesins in prophase requires a modification or a conformational change that at the same time protects these soluble complexes from recognition by the separin-dependent cleavage pathway.

Is Separin a Protease and How Is It Regulated?

The discovery that SCC1 is cleaved in a separin-dependent manner in both budding yeast (Uhlmann et al., 1999) and in humans (this study) begs the question as to whether separin is a protease or is an activator of such an enzyme. The simplest interpretation of our observation that separin immunoprecipitates are sufficient to cleave the SCC1 subunit of purified cohesin complexes (Figure 6) is that separin itself is the SCC1 protease, although we cannot yet exclude the possibility that a minor protease not detectable by Coomassie staining is present in our immunoprecipitates, or that separin functions as an activator of a protease associated with cohesin. Biochemical experiments by Uhlmann et al. (2000 [this issue of *Cell*]) indicate that the budding yeast separin Esp1p is a protease. Furthermore, the C-terminal domain of Esp1p which is highly conserved among separins from different species contains distant similarity to the catalytic site of cysteine endopeptidases of the CD family, and mutational analysis of this region has confirmed the importance of this region for Esp1p activity (Uhlmann et al., 2000 [this issue of *Cell*]). Separin therefore appears to be a protease.

If separin triggers anaphase, it is obvious that its activity has to be tightly regulated. In both budding and fission yeast, securin destruction seems to be required for separin activation (Cohen-Fix et al., 1996; Funabiki et al., 1996a, Funabiki et al., 1996b; Ciosk et al., 1998; Uhlmann et al., 1999). However, additional mechanisms must exist, because in budding yeast, the deletion of Pds1p does not change the timing of anaphase (Alexandru et al., 1999). Our experiments suggest that at least two reactions occur when separin is activated: securin dissociates from separin, presumably due to APC-mediated proteolysis (Zou et al., 1999), and separin is partially cleaved, resulting in the formation of two distinct C-terminal fragments (Figures 3B and 6A). Because securin has been shown to block anaphase in *Xenopus* extracts in vitro (Zou et al., 1999), it is likely that securin proteolysis is required for separin activation. It is less clear if separin cleavage is also required for separin activation, or if this reaction inactivates the enzyme. Intriguingly, separins belong to the same class of cysteine endopeptidases as caspases (Uhlmann et al., 2000 [this issue of *Cell*]), apoptotic proteases whose activities are known to be regulated by at least two mechnisms. Proteolytic cleavage is required for caspase activation, whereas the binding of inhibitor of apoptosis proteins (IAPs) inhibits cleavage (reviewed by Salvesen and Dixit, 1999). It is therefore attractive to speculate that securin proteolysis may enable separin cleavage, perhaps in an autocatalytic reaction, resulting in its activation. However, it is equally plausible that cleavage is a self-limiting mechanism that inactivates separin. Such an inactivation mechanism may be required to allow the rebinding of cohesin complexes to chromatin already in telophase. This hypothesis could xplain why in vertebrates cohesins rebind to chromatin already at the end of mitosis, whereas in budding yeast, where no Esp1p cleavage has yet been observed, separin remains active throughout G1, thus preventing the accumulation of cohesin complexes on chromatin until the G1-S transition (Uhlmann and Nasmyth, 1998).

Is Separin a Universal "Chromatid Separase" in All Eukaryotes?

In the accompanying article, Buonomo et al. (2000 [this issue of *Cell*]) show that Esp1p controls meiosis in budding yeast by cleaving Rec8p, a cohesin that replaces Scc1p/Mcd1p in meiotic cells. In fission yeast, a noncleavable version of the SCC1 ortholog Rad21p has recently also been found to block mitotic sister separation (Tomonaga et al., 2000). Our and these results together suggest that separin functions as a sister chromatid separase in all eukaryotes, an enzyme whose existence has been discussed by cytologists for 27 years already (Ostergren and Andersson, 1973; Sumner, 1992). To illustrate this function and the proteolytic activity of separin, and to distinguish its name better from its binding partner securin, we propose to call this enzyme separase in the future.

Experimental Procedures

cDNAs and Antibodies

The human separin cDNA KIAA0165 (Nagase et al., 1996) and a human securin expression plasmid were kindly provided by Kazusa DNA Research Institute, Japan and Michael Gmachl, Vienna, respectively. Murine SCC1 was amplified from a Swiss 3T3 cell cDNA library by polymerase chain reactions.

Mouse monoclonal (7A6) and rabbit polyclonal separin antibodies were raised against a recombinant C-terminal fragment of human separin expressed in *E. coli*. Polyclonal mouse and rabbit antibodies were raised against full-length recombinant human securin. Polyclonal antibodies were raised against the C terminus (YSDIIATPGP RFH) or against the N terminus of human SCC1 (FHDFDQPLPDLDDI DVAQQFSLNQSRVEEC). Either mouse monoclonal (9E10) or rabbit polyclonal (Gramsch Laboratories, Schwabhausen, Germany) myc-epitope antibodies were used. All antibodies were affinity purified. All other antibodies have been described (Kramer et al., 2000; Sumara et al., 2000).

Cells, Cell Culture, and Synchronization

For the generation of a HeLa cell line stably expressing SCC1, murine SCC1 was C-terminally tagged with 9 myc-epitopes and inserted into pHTet-Hygro (pUHD10–3; Gossen and Bujard, 1992, with the hygromycin resistance gene from pBabe Hygro; Morgenstern and Land, 1990). The resulting plasmid was transfected into HeLa Tet-off cells by electroporation. Selection with 200 μg/ml hygromycin B was started after 37 hr. After 2 weeks, cell lines arising from single cells were picked and tested for SCC1-myc expression by immunofluorescence microscopy. SCC1-myc expressing cells were further subcloned and cultured in the presence of 200 μg/ml hygromycin B.

HeLa cells were synchronized by a thymidine block as described (Kramer et al., 2000) and released into medium with or without 330 nM nocodazole.

For nocodazole release experiments, logarithmically proliferating HeLa cells were first grown to about 60% confluency prior to addition of 330 nM nocodazole for 18 hr. Subsequently, cells were washed twice with PBS and seeded into fresh medium. Synchronization and cell cycle state were examined by propidiumiodide staining and FACS analysis.

Immunofluorescence Microscopy

Cells were grown on glass coverslips, washed with PBS, extracted in 0.1% Triton X-100 in PBS for 3 min, washed with PBS, and fixed

in 4% paraformaldehyde/PBS for 15 min. Subsequently, cells were washed with PBS, incubated in 50 mM NH_4Cl/PBS for 5 min, washed with PBS, and incubated in 0.1% Triton X-100/PBS for 5 min followed by one further wash with PBS. Cells were incubated in 3% BSA/PBS for 2 hr prior to incubation with rabbit anti-myc antibody followed by Alexa Fluor 568–labeled goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR), human CREST serum (kind gift from Georg Krohne, Würzburg), and Alexa Fluor 488–labeled goat anti-human IgG (Molecular Probes, Eugene, OR). All antibody incubations were performed for one hour in blocking buffer, and cells were washed three times in PBS between different incubations. DNA was counterstained with DAPI (Roche Diagnostics, Mannheim, Germany). Coverslips were mounted in Slow Fade Light (Molecular Probes) and examined with a Zeiss Axioplan microscope fitted with appropriate fluorescence filters and equipped with a CCD-camera (Princeton Instruments Inc., Tucson, AZ). The capture time for Alexa Fluor 568 was standardized to 3 s with a 100× objective. Images were processed using MetaMorph Software (Universal Imaging Corp., West Chester, PA).

Cell Extracts and Fractionation

Xenopus egg extracts were obtained as described (Vorlaufer and Peters, 1998). For the generation of mitotic extracts, interphase extracts were preincubated for 30 min at room temperature with a Δ90 fragment of sea urchin cyclin B (Murray et al., 1989). Extracts from HeLa cells containing 6–10 mg/ml protein were prepared in immunoprecipitation (IP) buffer (20 mM Tris [pH 7.7], 100 mM NaCl, 20 mM β-glycerophosphate, 5 mM $MgCl_2$, 1 mM NaF, 0.1% Triton X-100, 10% glycerol, 1 mM DTT, and protease inhibitors [leupeptin, chymostatin, and pepstatin at a concentration of 10 μg/ml]). Cells were lysed for 20 min on ice by using a glass-teflon potter. To obtain crude chromatin and soluble protein fractions, total HeLa extracts were centrifuged 5 to 10 min at 4°C at 13, 000 rpm using a table top centrifuge. For sedimentation analysis, 100,000 × g supernatant of HeLa SCC1-myc cells containing 1.4 mg of protein was centrifuged for 18 hr at 30,000 rpm and 4°C through a 5%–20% sucrose gradient (in IP-buffer containing 5% glycerol) using a SW40 rotor (Beckman).

SCC1-myc Cleavage in *Xenopus* Extracts

Extracts from nocodazole-arrested HeLa SCC1-myc cells containing 250 μg of protein were centrifuged in a taple top centrifuge for 5 min at 13,000 rpm and 4°C. Either the chromatin-containing pellet resuspended in 5 μl XB (20 mM HEPES [pH 7.7], 100 mM KCl, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$) plus 0.005% Triton X-100, or 12.5 μl of the supernatant was mixed with 25 μl interphase or mitotic *Xenopus* extract and incubated at room temperature. Aliquots of 4 μl were taken at different time points and analyzed by SDS-PAGE and immunoblotting. To immunodeplete APC and separin, Affi-Prep Protein A beads (Bio-Rad, Hercules, CA) were loaded with affinity-purified polyclonal CDC27 or separin antibodies (1–2 μg antibodies/μl beads). Cell extracts were depleted for 1 hr on ice using an extract to bead ratio of 1.5–2 to 1.

SCC1 Cleavage Using Separin Immunoprecipitates

Cohesins were immunopurified from nocodazole-arrested HeLa SCC1-myc cells as described (Sumara et al., 2000). Separin was immunoprecipitated from low speed supernatant of nocodazole-arrested HeLa cells using Affi-Prep protein A beads to which 1.5 μg antibodies/μl beads had been coupled (100 μl beads were used for extract containing 20 mg protein). For Coomassie staining and immunoblot analyses of the immunoprecipitates, 50 μl beads were washed with TBS-TX (TBS plus 0.5 mM DTT, 500 mM NaCl, and 0.5% Tween 20), 45 μl were used for Coomassie staining and 5 μl for immunoblotting. For SCC1-myc cleavage assays, the immunoprecipitates were washed several times with XB supplemented with 0.5 mM DTT and 0.1% Triton X-100 (the detergent was omitted in the last two washes). Subsequently, the immunoprecipitates were incubated with 6 volumes of buffer (1 hr on ice), or interphase or mitotic *Xenopus* extracts (1 hr at room temperature) and then washed either 4 times in XB plus 0.5 mM DTT or 6 times in TBS-TX. Subsequently, 25 μl beads were incubated with 30 μl cohesin mixture (14 μl purified cohesin diluted in XB containing 10 mM $MgCl_2$, 1 mM EGTA, and 1 mM ATP) in a thermomixer at 22°C and 1,200 rpm. At different time points, 6 μl aliquots were taken and analyzed by immunoblotting. In some experiments, APC activity in the mitotic *Xenopus* extracts was inhibited by addition of 1 mM of N-terminal human cyclin B peptides comprising either the wild-type or a mutated version of the destruction box (kind gift of E. Kramer, Vienna).

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References

Alexandru, G., Zachariae, W., Schleiffer, A., and Nasmyth, K. (1999). Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. EMBO J. *18*, 2707–2721.

Amon, A. (1999). The spindle checkpoint. Curr. Opin. Genet. Dev. *9*, 69–75.

Brenner, S., Pepper, D., Berns, M.W., Tan, E., and Brinkley, B.R. (1981). Kinetochore structure, duplication, and distribution in mammalian cells: analysis by human autoantibodies from scleroderma patients. J. Cell Biol. *91*, 95–102.

Buonomo, S.B.C., Clyne, R.K., Fuchs, J., Loidl, J., Uhlmann, F., and Nasmyth, K. (2000). Disjunction of homologous chromosomes in meiosis I depends on proteolytic cleavage of the meiotic cohesin Rec8 by separin. Cell *103*, this issue, 387–398.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M., and Nasmyth, K. (1998). An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. Cell *93*, 1067–1076.

Cohen-Fix, O., Peters, J.M., Kirschner, M.W., and Koshland, D. (1996). Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. Genes Dev. *10*, 3081–3093.

Darwiche, N., Freeman, L.A., and Strunnikov, A. (1999). Characterization of the components of the putative mammalian sister chromatid cohesion complex. Gene *233*, 39–47.

Dej, K.J., and Orr-Weaver, T.L. (2000). Separation anxiety at the centromere. Trends Cell Biol. *10*, 392–399.

Funabiki, H., Yamano, H., Kumada, K., Nagao, K., Hunt, T., and Yanagida, M. (1996a). Cut2 proteolysis required for sister-chromatid seperation in fission yeast. Nature *381*, 438–441.

Funabiki, H., Kumada, K., and Yanagida, M. (1996b). Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. EMBO J. *15*, 6617–6628.

Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA *89*, 5547–5551.

Guacci, V., Hogan, E., and Koshland, D. (1994). Chromosome condensation and sister chromatid pairing in budding yeast. J. Cell Biol. *125*, 517–530.

Guacci, V., Koshland, D., and Strunnikov, A. (1997). A direct link

between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD1 in S. cerevisiae. Cell *91*, 47–57.

Holloway, S.L., Glotzer, M., King, R.W., and Murray, A.W. (1993). Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. Cell *73*, 1393–1402.

Koshland, D.E., and Guacci, V. (2000). Sister chromatid cohesion: the beginning of a long and beautiful relationship. Curr. Opin. Cell Biol. *12*, 297–301.

Kramer, E.R., Scheuringer, N., Podtelejnikov, A.V., Matthias, M., and Peters, J.M. (2000). Mitotic regulation of the APC activator proteins CDC20 and CDH1. Mol. Biol. Cell *11*, 1555–1569.

Leismann, O., Herzig, H., Heidmann, S., and Lehner, C.F. (2000). Degradation of Drosophila PIM regulates sister chromatid separation during mitosis. Genes Dev. *14*, 2192–2205.

Losada, A., Hirano, M., and Hirano, T. (1998). Identification of Xenopus SMC protein complexes required for sister chromatid cohesion. Genes Dev. *12*, 1986–1997.

May, G.S., McGoldrick, C.A., Holt, C.L., and Denison, S.H. (1992). The bimB3 mutation of Aspergillus nidulans uncouples DNA replication from the completion of mitosis. J. Biol. Chem. *267*, 15737–15743.

Michaelis, C., Ciosk, R., and Nasmyth, K. (1997). Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell *91*, 35–45.

Morgenstern, J.P., and Land, H. (1990). Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. *18*, 3587–3596.

Murray, A.W., Solomon, M.J., and Kirschner, M.W. (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature *339*, 280–286.

Nagase, T., Seki, N., Ishikawa, K., Tanaka, A., and Nomura, N. (1996). Prediction of the coding sequences of unidentified human genes. V. The coding sequences of 40 new genes (KIAA0161-KIAA0200) deduced by analysis of cDNA clones from human cell line KG-1 (supplement). DNA Res. *3*, 43–53.

Nasmyth, K., Peters, J.M., and Uhlmann, F. (2000). Splitting the chromosome: cutting the ties that bind sister chromatids. Science *288*, 1379–1385.

Ostergren, G., and Andersson, L. (1973). Staining the Kinetochore in Plant Chromosomes, Volume *23*, T. Caspersson and L. Zech, eds. (Stockholm: Nobel Foundation), pp. 159–161.

Peters, J.M. (1999). Subunits and substrates of the anaphase-promoting complex. Exp. Cell Res. *248*, 339–349.

Pidoux, A.L., and Allshire, R.C. (2000). Centromeres: getting a grip of chromosomes. Curr. Opin. Cell Biol. *12*, 308–319.

Rieder, C.L., and Cole, R. (1999). Chromatid cohesion during mitosis: lessons from meiosis. J. Cell Sci. *112*, 2607–2613.

Salvesen, G.S., and Dixit, V.M. (1999). Caspase activation: the induced-proximity model. Proc. Natl. Acad. Sci. USA *96*, 10964–10967.

Sigrist, S., Jacobs, H., Stratmann, R., and Lehner, C.F. (1995). Exit from mitosis is regulated by Drosophila fizzy and the sequential destruction of cyclins A, B and B3. EMBO J. *14*, 4827–4838.

Stratmann, R., and Lehner, C.F. (1996). Separation of sister chromatids in mitosis requires the Drosophila pimples product, a protein degraded after the metaphase/anaphase transition. Cell *84*, 25–35.

Sumara, I., Vorlaufer, E., Gieffers, C., Peters, B.H., and Peters, J.-M. (2000). Characterization of vertebrate cohesin complexes and their regulation in prophase. J. Cell. Biol., in press.

Sumner, A.T. (1991). Scanning electron microscopy of mammalian chromosomes from prophase to telophase. Chromosoma *100*, 410–418.

Sumner, A.T. (1992). Inhibitors of topoisomerases do not block the passage of human lymphocyte chromosomes through mitosis. J. Cell Sci. *103*, 105–115.

Tang, T.T.L., Bickel, S.E., Young, L.M., and Orr-Weaver, T.L. (1998). Maintenance of sister-chromatid cohesion at the centromere by the Drosophila MEI-S332 protein. Genes Dev. *12*, 3843–3856.

Tomonoga, T., Nagao, K., Kawasaki, Y., Furuya, K., Murakami, A., Morishita, J., Yuasa, T., Sutani, T., Kearsey, S., Uhlmann, F., et al. (2000). Characterization of fission yeast cohesin: essential anaphase proteolysis of Rad21 phosphorylated in the S phase. Genes Dev., in press.

Toth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleiffer, A., and Nasmyth, K. (1999). Yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. Genes Dev. *13*, 320–333.

Uhlmann, F., and Nasmyth, K. (1998). Cohesion between sister chromatids must be established during DNA replication. Curr. Biol. *8*, 1095–1101.

Uhlmann, F., Lottspeich, F., and Nasmyth, K. (1999). Sister chromatid separation at anaphase onset is triggered by cleavage of the cohesin subunit Scc1p. Nature *400*, 37–42.

Uhlmann, F., Wernic, D., Poupart, M.-A., Koonin, E.V., and Nasmyth, K. (2000). Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast. Cell *103*, this issue, 375–386.

Vorlaufer, E., and Peters, J.M. (1998). Regulation of the cyclin B degradation system by an inhibitor of mitotic proteolysis. Mol. Biol. Cell *9*, 1817–1831.

Yanagida, M. (2000). Cell cycle mechanisms of sister chromatid separation; roles of Cut1/separin and Cut2/securin. Genes Cells *5*, 1–8.

Zou, H., McGarry, T.J., Bernal, T., and Kirschner, M.W. (1999). Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science *285*, 418–422.

Note Added in Proof

Results showing that the cohesin subunit SA1 can be detected between sister chromatids of mitotic chromosomes assembled in *Xenopus* extracts have been published recently: Losada, A., Yokochi, T., Kobayashi, R., and Hirano, T. (2000). J. Cell Biol. *150*, 405–416.

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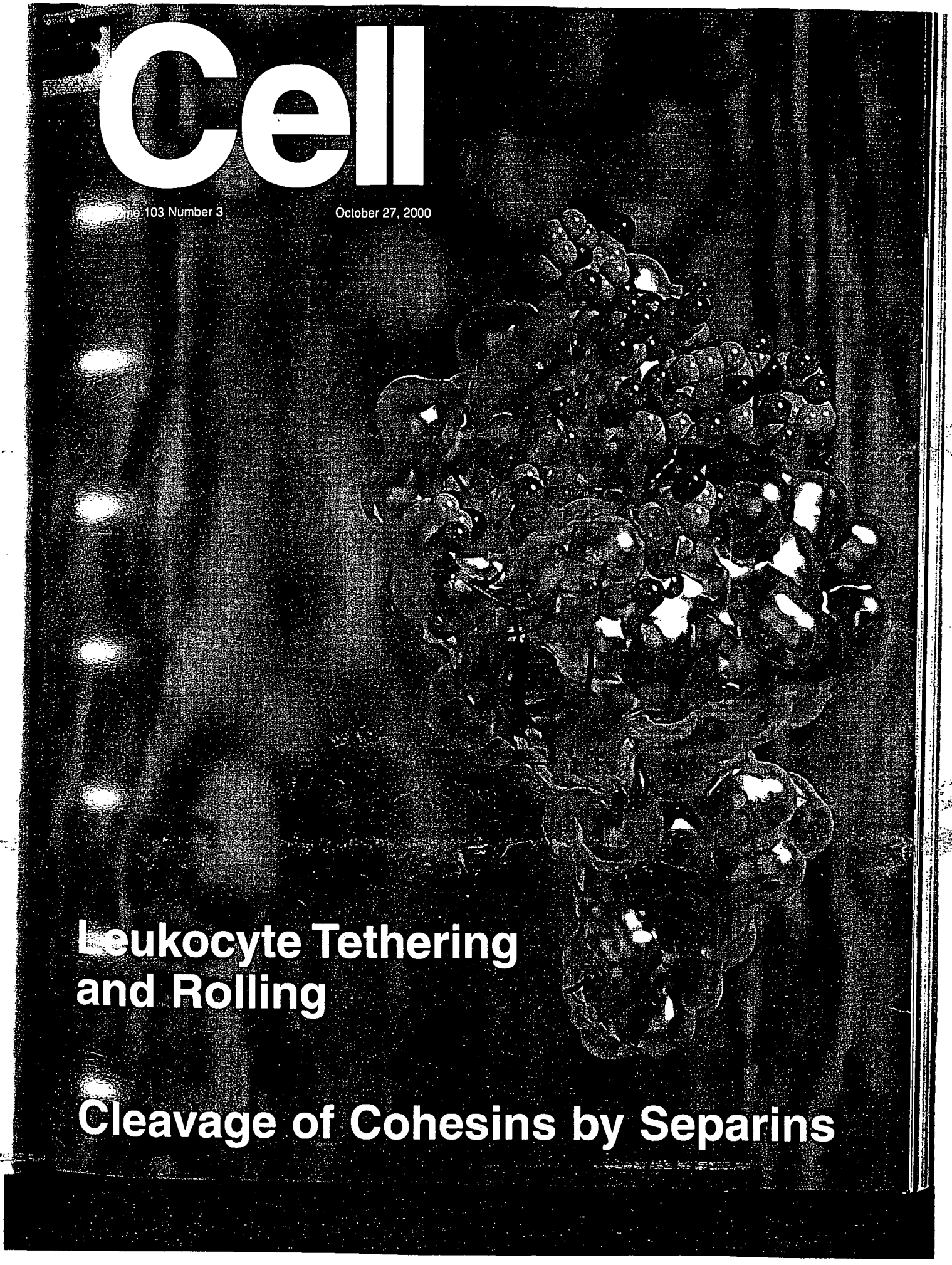

Leukocyte Tethering and Rolling

Cleavage of Cohesins by Separins

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(54) Title: INHIBITORS OF SEPARIN, METHOD FOR IDENTIFYING THEM AND USES

(57) Abstract

Method for identifying compounds that interfere with or modulate sister chromatid separation in animal or plant cells by modulating a protease with separin–like cysteine endopeptidase activity. Inhibitors of separin activity are useful in cancer therapy, to prevent birth defects and to increase ploidy in plants.

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Title: INHIBITORS OF SEPARIN, METHOD FOR IDENTIFYING THEM AND USES

The invention relates to compounds influencing mitosis and meiosis in eukaryotic cells and methods for identifying such compounds. In particular, the invention largely relates to the treatment and prevention of human conditions by modulating sister chromatid segregation.

During the process of cell division, sister chromatids are pulled to opposite halves of the cell by microtubules emanating from spindle poles at opposite sides of the cell. One set of microtubules inter-digitates with others emanating from the opposite pole. Their role is to keep (and drive) the two poles apart. Meanwhile, a second set of microtubules attaches to chromosomes via specialized structures called kinetochores and pulls them towards the poles. Sister chromatids segregate away from each other because their kinetochores attach to microtubules emanating from opposite poles (Rieder et al., 1998). Chromosomes are not mere passengers during this process. During metaphase, the tendency of microtubules to move sisters apart is counteracted by cohesion holding sisters together. Cohesion therefore generates the tension by which cells align sister chromatids on the metaphase plate. Were sisters to separate before spindle formation, it is difficult to imagine how cells could distinguish sisters from chromatids that were merely homologous. The sudden loss of cohesion, rather than an increase in the exertion of microtubules, is thought to trigger sister separation during anaphase (Miyazaki et al., 1994). Cohesion also prevents chromosomes falling apart due to double strand breaks and facilitates their repair using recombination.

To avoid missegregation of chromosomes, anaphase must only be initiated after sister chromatids of each duplicated chromosome have attached to opposite poles of the mitotic spindle. Microtubules are thought to "find" kinetochores by a "search and capture" mechanism which cannot be completed simultaneously for all chromosomes (Hayden et al., 1990; Merdes and De Mey, 1990). Cells therefore possess regulatory mechanisms that delay sister chromatid separation until the last chromosome has achieved bipolar attachment. The dissolution of sister chromatid cohesion at the metaphase to anaphase transition is therefore a highly regulated step during the eukaryotic cell cycle.

Sister chromatid cohesion depends on a multi-subunit complex called cohesin (Losoda et al., 1998), which contains at least four subunits: Smc1p, Smc3p, Scc1p, and Scc3p, all of which are conserved between yeast and humans. It is likely, but not yet proven, that cohesin is a key constituent of the tether that holds sister chromatids together. The association between cohesin and chromosomes has recently been shown to depend on the Scc2 (Mis4) protein. Cohesion is established during DNA replication (Uhlmann and Nasmyth, 1998). It has been recently shown that the Eco1 (Ctf7) protein is required for the establishment of cohesion during S phase but unlike cohesin is not required to maintain cohesion during G2 and M phases. In yeast, cohesin remains tightly associated with chromosomes until metaphase; that is, it is present on chromosomes during their alignment during metaphase. In animal cells, however, the bulk of cohesin dissociates from chromosomes during prophase (Losada et al., 1998). It is unclear how much cohesin, if any, remains on chromosomes during metaphase. The nature of the link that holds sister chromatids together during metaphase in animal cells is therefore unclear. It could either involve a small fraction of cohesin that remains associated with chromosomes or some other protein complex.

In yeast, at least two of cohesin's subunits, Scc1p and Scc3p, suddenly disappear from chromosomes at precisely the point at which sister chromatids separate (Michaelis et al., 1997). This has led to the notion that a sudden change in the state of cohesin might trigger the onset of anaphase, at least in yeast. In Drosophila, the meiS332 protein, which binds to chromosomes during prometaphase, also disappears at the onset of anaphase. MeiS332 is required for sister chromatid cohesion during meiosis but not during mitosis (Moore et al., 1998; Kerrebrock et al., 1995). MeiS332 is probably not a cohesin subunit nor is it apparent whether homologous proteins exist in humans.

Both the dissociation of Scc1p from chromosomes and the separation of sister chromatids are dependent on a specialized sister separating protein (a separin) called Esp1p (Ciosk et al., 1998). Separins homologous to Esp1 exist in the fission yeast Schizosaccharomyces pombe, in the fungus Aspergillus nidulans, in the nematode worm Caenorhabditis elegans, the fruit fly Drosophila melanogaster, in the frog Xenopus laevis, in the plant

Arabidopsis thaliana, and in man. This strongly suggests that separins have a fundamental role in chromosome segregation that is conserved between plants, fungi, and animals. Esp1 is tightly bound by an inhibitory protein called Pds1p whose destruction shortly before the metaphase to anaphase transition is triggered by ubiquitination mediated by the anaphase promoting complex (APC) (Cohen-Fix et al., 1996). The APC is large multi-subunit complex, most of whose subunits are conserved between yeast and humans. Together with activator proteins called Cdc20p and Cdh1p, it mediates the ubiquitination and thereby destruction of many different cell cycle proteins, including anaphase inhibitors like Pds1 and mitotic cyclins. Pds1 destruction is mediated by a form of the APC bound by the activator Cdc20. This form is called APC^{Cdc20}. (For a review, see Peters, 1998)

Proteins with similar properties to Pds1 have been found in fission yeast (Cut2p), in Xenopus, and in humans (Funabiki et al., 1996; Zou et al., 1999). The APC is essential for sister chromatid separation in most if not all eukaryotic organisms. In yeast, it is clear that its main role in promoting sister separation is to destroy Pds1, which liberates Esp1 and allows it to destroy sister chromatid cohesion, possibly by destroying the physical links between sisters mediated by cohesin.

It was an object of the invention to further eluciate the mechanism of sister chromatid separation.

In particular, it was an object of the invention to understand the mechanism by which Esp1p mediates the dissocation of Scc1p from chromosomes in the budding yeast in order to take advantage of this mechanism by using it as a target in human therapy, in particular of cancer therapy, and as a target in all other situations where modulation of sister chromatid separation is therapeutically or otherwise beneficial.

To solve the problem underlying the present invention, the following approach was taken:

The observation that the dissociation of Scc1p from chromosomes at the onset of anaphase is dependent on Esp1 suggested that Esp1 might either have a direct role in this process or that Esp1 might be indirectly involved

by initiating a process that leads to Scc1p's dissociation. It was found in experiments of the present invention that Esp1 also prevents association of Scc1p with chromosomes during G1 (see Example 1), which strongly suggests that Esp1's role might be very direct. Scc1p is an unstable protein which is rapidly destroyed following its dissociation from chromosomes at the onset of anaphase and which must be re-synthesised during late G1 during the next cell cycle in order for cohesion to be established at the next round of DNA replication (Michaelis et al., 1997) It was found that Scc1p synthesised during G2 is also capable of binding to yeast chromosomes but that it fails to produce cohesion under these circumstances (Uhlmann and Nasmyth, 1998). However, it was noted that Scc1p synthesised during early G1 binds to chromosomes poorly, if at all. As shown in Figure 1, inactivation of Esp1 permits the efficient association between Scc1p and chromosomes during early G1. The implication is that Esp1 not only triggers Scc1p's dissocation from chromosomes at the onset of anaphase but also prevents Scc1p's stable association with chromosomes during the subsequent G1 period. This strongly suggests that Esp1 has a fairly direct role in controlling the association between Scc1p and chromosomes.

Starting from this finding, an assay was established by which Esp1 activity could be measured in vitro. A crude preparation of yeast chromatin isolated from cells arrested in a metaphase-like state by nocodazole, was incubated with a soluble extract prepared from cells over-producing Esp1 from the GAL promoter (Figure 2). To detect Scc1p, cells were used whose Scc1 protein was tagged at its C-terminus with multiple HA or Myc epitopes, which can readily be detected with monoclonal antibodies. About 70% of the total Scc1p in nocodazole blocked cells is tightly associated with chromatin and is therefore present in the chromatin fraction that was used. Most of the Scc1p remains tightly associated with chromatin following incubation with an extract prepared from esp1-1 mutant cells but most disappears from the chromatin fraction upon incubation with extracts containing high levels of wild type Esp1 protein. Somewhat surprisingly, the Scc1p protein induced to dissociate from chromatin by Esp1 appeared in the "soluble" supernatant fraction as a cleaved product. The C-terminal fragments of this cleavage were detected by using as a substrate a C-terminally tagged Scc1 protein and N-terminal fragments using as substrate an N-terminally tagged Scc1 protein. The sizes of these cleavage products

suggested that Esp1 induces one or more specific cleavages of Scc1p within a 10 kd interval. This Esp1-dependent cleavage was inhibited by the addition of reticulate lysate that had translated Pds1 but not by an otherwise identical lysate that had translated an unrelated control protein. The Esp1 activity detected by the cleavage assay is therefore inhibited by Pds1, which demonstrates directly, for the first time, that Pds1 is indeed an inhibitor of Esp1p.

To address whether Esp1 induced cleavage of Scc1p also occurs in vivo at the onset of anaphase, a yeast strain was constructed in which expression of the APC activator Cdc20p is under control of the galactose inducible *GAL1-10* promoter. The strain also expressed an Scc1 protein tagged at its C-terminus with multiple HA or myc epitopes. Cells from this strain were arrested in metaphase by incubation in galactose free medium and then induced to embark on anaphase highly synchronously by the addition of galactose. Figure 3 shows that sister chromatids separate in most cells within 15 minutes and that Scc1p dissociates from chromosomes with similar if not identical kinetics. A low level of an Scc1 cleavage product was detected that is identical to that seen in vitro in cycling cells but none in cells arrested in metaphase. The cleavage product suddenly appeared upon induction with galactose with kinetics that were similar if not identical to the separation of sister chromatids and dissocation of Scc1p from chromosomes. To establish whether this in vivo cleavage was dependent on Esp1 activity the extent of Scc1p cleavage in wild type and esp1-1 mutants when released from cdc20 arrest at 35.5°C was compared (the restrictive temperature for esp1-1). The extent of Scc1p cleavage was greatly reduced in the esp1-1 mutant. It was concluded that Esp1 promotes the cleavage of Scc1p and its dissociation from chromosomes both in vivo and in vitro.

To address whether cleavage of Scc1 mediated by Esp1 is important either for sister chromatid separation or for Scc1p's dissociation from chromosomes, the cleavage site was mapped in order that it could then be mutated. An epitope tagged Scc1p protein from cells that had been stimulated to undergo anaphase by induction of Cdc20 expression was immunoprecipiated and the immunoprecipitated proteins were separated on SDS page. A short stretch of N-terminal amino acid sequence from the

C-terminal cleavage fragment was then determined by Edman degradation. This showed that cleavage in vivo had occured between a pair of arginines at positions 268 and 269. The N-terminal of these arginine residues was then mutated to aspartic acid and an HA tagged version then was expressed from the *GAL1-10* promoter in yeast cells whose endogenous Scc1 protein was myc tagged. Galactose induced expression of this single mutant protein had little effect on cell proliferation. To establish whether the mutation had indeed abolished cleavage, chromatin from cells expressing the mutant protein was isolated and used as a substrate in the Esp1 assay. This showed that cleavage at site 268 was indeed eliminated by the aspartic acid mutation. However, the mutated protein was still cleaved in an Esp1-dependent manner. The C-terminal cleavage product from the mutant protein was about 10 kDa longer than that from wild type. The interpretation of these results is that Scc1p is actually cleaved at two sites approximately 10 kDa apart. Cleavage at the more C-terminal site is highly efficient, which is why C-terminal tagged proteins cleaved only at the more N-terminal site were rarely detected.

To identify the second cleavage site, sequences within Scc1p which are similar in sequence to those surrounding the known C-terminal cleavage site were looked for. A 5 out of 7 amino acid match at position 180 found. Furthermore, the distance between this potential site and the known cleavage site is consistent with the greater length of the cleavage product generated in vitro from protein whose C-terminal site (R268) had been mutated. The matching sequence also contained a pair of arginines and therefore the more N-terminal arginine was mutated to aspartic acid. Next the effect of expressing HA tagged versions of wild type Scc1p, both single mutant proteins, and the double mutant protein from the GAL1 promoter in yeast was compared. As a host for these studies a strain was used whose endogenous Scc1p was myc tagged. Neither wild type nor either single mutant blocked cell proliferation but expression of the double mutant protein was lethal. Chromatin from cells transiently expressing these proteins was prepared and it was shown that HA tagged double mutant protein was no longer cleaved when incubated in Esp1-containing extracts while the myc tagged wild type protein was efficiently cleaved.

To investigate why cells expressing a non-cleavable Scc1p protein (the R180D R268D double mutant) cannot proliferate, centrifugal elutriation was used to isolate G1 cells from a culture growing in the absence of galactose , which were then incubated in the presence and absence of galactose (Fig. 4). In order to minimize the duration of mutant protein expression, the cells grown in the presence of galactose were transferred to glucose containing medium after most cells had replicated their DNA (at 135 min). In the absence of galactose, sister separation and dissociation from chromosomes of endogenous myc tagged Scc1p occured simultaneously, approximately 60 min after DNA replication. Transient expression of double mutant protein greatly reduced sister chromatid separation (Fig. 4b) but did not affect dissociation of endogenous myc tagged wild type protein (Fig. 4c and d). Furthermore, the mutant protein remained tightly associated with chromosomes long after the endogenous wild type protein had disappeared. Expression of the mutant protein did not greatly delay cell cycle progression and most cells underwent cytokinesis, producing progeny with low (0-0.5C) amounts of DNA and cells with less than a 2C DNA content (Fig. 4a). The dissociation from chromosomes of wild type protein on schedule shows that the lack of sister separation in cells expressing non-cleavable Scc1p is not due to a lack of Esp1 activity. Collectively, the data obtained imply that cleavage of Scc1p at one of two sites is necessary both for sister chromatid separation and for dissociation of Scc1p from chromosomes.

From the obtained results it can be concluded that cohesin directly mediates the link between sister chromatids that is established during DNA replication and is maintained until metaphase. It can be further concluded that Esp1's activation by proteolysis of Pds1 (and by as yet to be identified other mechanisms) generates an activity inside cells that cleaves the Scc1p subunit of cohesin and that this event both destroys sister chromatid cohesion and causes Scc1p and possibly other cohesin subunits to dissociate from chromosomes.

From the above results it is clear that sister chromatid separation depends on cleavage of chromosome-bound Scc1 by an Esp1-dependent proteolytic activity that appears in cells at the onset of anaphase. It was next asked whether Scc1 as an isolated protein (rather than in the chromosomal

context) can also serve as a substrate for the Esp1-dependent cleavage reaction. Fig. 5 describes the purification of recombinant Scc1 after over-expression in insect cells infected with a recombinant baculovirus. Scc1 was purified either from an asynchronously growing population of infected insect cells (Fig. 5a, lanes 1-4) or from infected insect cells that had been treated with the phosphatase inhibitor okadaic acid. Treatment with okadaic acid induces a metaphase-like state within the insect cells as a consequence of which Scc1 is obtained in a mitotically phosphorylated form (Fig. 5a, lane 5). Scc1 in yeast appears also phosphorylated in a mitosis specific manner. These Scc1 fractions, that were more than 90% pure as judged by SDS-PAGE followed by staining of the gel with Coomassie brilliant blue, were then used in the Scc1 cleavage assay as described above (Fig. 5b). Both unphosphorylated and phosphorylated purified Scc1 were cleaved in an Esp1-dependent manner in vitro, however, the efficiency of cleavage was much greater when Scc1 was in the mitotically phosphorylated state. From this experiment it was concluded that isolated Scc1 which is neither part of the cohesin compex nor bound to chromosomes is a substrate for cleavage by Esp1, at least if it is in its mitotically phosphorylated state.

It was then addressed, whether Esp1 is itself the protease that cleaves Scc1. Inhibitor studies showed that the in vitro cleavage activity could be inhibited by N-ethyl maleimide an inhibitor specific for proteases using a catalytic cysteine residue. Inspection of the amino acid sequence within the evolutionary conserved C-terminal half of Esp1 revealed that exactly one cysteine and one histidine residue are conserved in all known separin homologues. These two residues might therefore form the catalytic dyad of a new subclass of cysteine protease. When the amino acid sequences surrounding the potential catalytic dyad were further analysed, it was found that both the cysteine and the histidine residues are preceded by a sequence stretch predicted to form a hydrophobic beta sheet. Furthermore, the histidine is invariably flanked by two glycine residues and the cysteine is preceded by a glycine providing the possibility for a tight turns before or after the catalytic residues. This arrangment of histidine and a cysteine catalytic dyad residues fixed at the ends of two neighbouring strands of hydrophobic beta sheet is used in the caspase family of proteases and it seems likely that the same arrangment is used in separins like Esp1.

To provide evidence that Esp1 indeed uses these two amino acid residues histidine (amino acid position 1505) and cysteine (position 1531) as a catalytic dyad for cleaving Scc1,either of these amino acids were mutated to alanine. Both mutations completely abolished the proteolytic activity in yeast extracts after overproduction of the proteins (Fig. 6). Wild type Esp1 overexpressed to a similar level caused complete cleavage of the Scc1 substrate. It was concluded that histidine 1505 and cystein 1531 most likely form the catalytic dyad that provides Esp1 with its proteolytic activity to cleave Scc1.

Together these results provide compelling evidence that a proteolytic reaction in which Esp1 separin cleaves the cohesin Scc1 is the initiating event for sister chromatid separation at the metaphase to anaphase transition in mitosis in S. cerevisiae.

It was next asked whether the same proteolytic mechanism might act to initiate chromosome separation during the two meiotic nuclear divisions. During premeiotic DNA replication a Scc1-homolog, called Rec8, replaces Scc1 in the cohesin complex (Klein et al., 1999). Rec8, like Scc1, contains two separin recognition sites, which suggests that Esp1/separin might cleave Rec8 during meiosis to initiate meiotic chromosome separation. To test this, both separin cleavage sites within Rec8 were mutated to produce a non-cleavable version of this protein. Expression of the non-cleavable Rec8 during meiosis led to a block of the first meiotic nuclear division (Fig. 7A), indicating that cleavage of Rec8 is necessary to separate sister chromatid arms in the first meiotic division. When meiosis was followed in a yeast strain containing the *esp1-2* mutation, a temperature sensitive mutation in the ESP1 gene, a temperature dependent block of the first meiotic nuclear division was likewise observed (Fig. 7B). It was concluded that separin cleaves the cohesin Rec8 during the meiotic nuclear divisions as it cleaves Scc1 during the mitotic division.

The sequences of human homologs of budding yeast Esp1, Pds1 and Scc1 already exist in public databases. The human homologs of Esp1 and Pds1 are referred to as separin (Nagase et al., 1996; protein sequence: NCBI Acc. No. BAA11482; DNA sequence: NCBI Acc. No. D79987) and securin (Zou et al., 1999, Dominguez et al., 1998) respectively, and the human homolog of Scc1 as SCC1 (McKay et al., 1996; DNA sequence: NCBI Acc.

No. X98294; protein sequence: NCBI Acc. No. CAA 66940). In animal cells it has been shown that the majority of SCC1 dissociates from chromatin in prophase long before sister chromatids are separated in anaphase, and no cleavage of SCC1 has been observed during this process (Losada et al., 1999).

Another object of the experiments of the present invention was to test whether some SCC1 remains bound to condensed chromosomes and maintains sister chromatid cohesion until the initiation of anaphase, and to analyze whether the chromosome-bound form of SCC1 was subject to proteolytic cleavage at the onset of anaphase.

To answer these questions, the following approach was taken: Human HeLa cells were enriched in interphase by logarithmic growth and in metaphase by treatment with nocodazole, and crude chromatin and supernatant fractions were generated by differential centrifugation and analyzed for the presence of SCC1 by quantitative immunoblotting (Fig. 8). The amount of the total cellular SCC1 associated with chromatin was reduced from 56 % in logarithmically growing cells to 13 % in cells arrested in metaphase. It was concluded that most but not all SCC1 dissociates from chromatin before metaphase, consistent with the possibility that SCC1 may be required to maintain sister chromatid cohesion until the onset of anaphase.

To address whether the form of SCC1 that is associated with chromosomes in metaphase is cleaved in anaphase, HeLa cells were arrested at the onset of S-phase by double-thymidine treatment and were synchronously released into the cell cycle. Progression through the cell cycle was monitored at different time points after the release by analysis of the DNA content with fluorescence activated cell sorting (FACS) and by analyzing total cell lysates in immunoblot experiments. Figure 9 shows that a putative SCC1 cleavage product corresponding to 100 kDa was recognized by antibodies specific for the C-terminus of SCC1. Importantly, this band appeared specifically when the HeLa cells went through anaphase, as judged by FACS analysis and the disappearance of securin, cyclin B and CDC20, proteins that are known to be degraded specifically in anaphase.

To confirm that the anaphase-specific 100 kDa band is a cleavage prodcut of SCC1 and not a non-specific crossreaction of the antibodies used, the following two experiments were performed: First, antibodies specific for the N-terminus of SCC1 were raised and used to analyze the HeLa cells cycle fractions by immunoblotting. A band of 25 kDa was recognized specifically in anaphase (Fig. 9), consistent with the interpretation that SCC1 is cleaved into an C-terminal 100 kDa and a N-terminal 25 kDa fragment. Second, a HeLa cell line stably expressing mouse SCC1 fused to a myc epitope tag at the C-terminus (SCC1-myc) was analyzed by cell synchronization as above. The amount of SCC1-myc expressed in these cells is less than 10% of endogenous SCC1 and the ectopic protein is entirely incorporated into 14S cohesin complexes (Fig. 10A). In synchronized cells, antibodies to the myc epitope recognize a band of the expected size (120 kDa) that appears in anaphase with similar kinetics as the 100 and 25 kDa bands recognized by SCC1 antibodies, demonstrating unambiguously that mammalian SCC1 is cleaved in anaphase (Fig. 10B). In addition, these immunoblots revealed a second anaphase-specific fragment of SCC1-myc, suggesting that SCC1 cleavage occurs at at least two sites (Fig. 10B bottom panel).

Biochemical experiments in *Xenopus* have shown that the initiation of anaphase depends on proteolysis of securin mediated by APC^{CDC20} (Zou et al., 1999). Like budding yeast Pds1 and Esp1, securin and separin form a complex, consistent with the hypothesis that APC^{CDC20}-dependent securin proteolysis activates separin. To address whether SCC1 cleavage depends on activation of APC^{CDC20} and subsequent securin proteolysis HeLa cells were synchronized by double-thymidine treatment and released into the cell cycle in the presence of nocodazole. Nocodazole is a drug known to indirectly cause the inhibition of APC^{CDC20} and thereby to arrest cells in metaphase (reviewed by Peters, 1998). Specific antibodies to human securin were raised and it was shown that securin was not degraded under these conditions (Fig. 11). Importantly, no cleavage of SCC1 could be observed in the presence of nocodazole. The effect of the drug was reversible because release of nocodazole arrested cells into anaphase correlated with degradation of securin and formation of the SCC1 cleavage products (Fig. 12). These results suggest that SCC1 cleavage depends on activation of APC^{CDC20} and are consistent with the

hypothesis that securin degradation and subsequent separin activation are required for SCC1 cleavage.

To analyze the regulation of SCC1 cleavage further and as a first step to develop a screening assay for inhibitors of this reaction an *in vitro* assay utilizing SCC1-myc and cell cycle extracts prepared from *Xenopus* eggs was established. These extracts can be manipulated to represent either a stable interphase state in which APC^{CDC20} is inactive or a stable mitotic state in which APC^{CDC20} is active and in which sister chromatid separation can occur *in vitro* (Murray et al., 1991) When chromatin isolated from HeLa cells stably expressing SCC1-myc was incubated in *Xenopus* extracts, cleavage of SCC1 at two distinct sites could be detected in the mitotic but not in the interphase extract, further confirming that APC^{CDC20} activity is required for this event (Fig. 13). In SDS-PAGE, the cleavage products formed *in vitro* comigrated with the cleavage products formed *in vivo*, suggesting that cleavage in the extract occurs at physiologically relevant sites. Importantly, some SCC1 cleavage was also observed when chromatin-free supernatant fractions from HeLa cells were mixed with mitotic extracts (Fig. 13). This demonstrates that soluble human SCC1 can be a substrate for cleavage and thus makes the development of a simplified chromatin-free cleavage assay for drug screening purposes feasible.

To map the more N-terminal cleavage site in SCC1 a series of N- and C-terminal deletion mutants was generated and the electrophoretic mobility of the truncated proteins was compared to the mobility of the N- and C-terminal cleavage products formed *in vivo* (Fig. 14). cDNAs encoding deletion mutants were generated by polymerase chain reactions (PCR) and recombinant S^{35}-labeled proteins were generated from the PCR products by *in vitro* transcription and translation. This analysis indicated that SCC1 is cleaved between amino acid residues 169 and 183. This site contains the sequence motif $ExxR^{172}$ which is conserved in many SCC1 homologs in different species and is also found in both N-terminal cleavage sites of budding yeast Scc1. Preliminary results using the same mapping strategy indicate that the C-terminal cleavage site in SCC1 is located around amino acid residue 450 where the motif ExxR is found again.

Based on the results of the experiments of the present invention it can be concluded that separin-dependent SCC1 cleavage is a mechanism that is conserved from budding yeast to humans and that the same mechanism most likely exists in all eukaryotic organisms. The findings obtained in experiments performed with budding yeast are therefore also valid in higher eukaryotic organisms, in particular in man.

The interpretation of the data obtained in the experiments of the present invention further provides evidence that Esp1/separin itself is the protease responsible for the cleavage of Scc1p/SCC1.

From the results obtained in the experiments of the invention, it may, inter alia, be concluded that Scc1p/SCC1 is the only subunit of the cohesion comples cleaved by Esp1/separin.This does, however, not exclude the possibility that other types of proteins, for example, other cohesion proteins or proteins which regulate mitotic spindles, might also be targets/substrates of separin. One way of addressing this question is to make a version of Scc1p that has one cleavage site replaced with a site for a foreign protease (with the other cleavage site removed). An example for a convenient protease to use is TEV protease (Daugherty et al., 1989), which has a very specific cleavage site (Glu-Asn-Leu-Tyr-Phe-Gln-Gly). A strain can be constructed that contains: the SCC1 gene containing a TEV protease cleavage site, a chromosomal *cdc20-3* mutation, and the TEV protease gene under *GAL1-10* inducible control. In the presence of galactose at the restrictive temperature (when *cdc20-3* cells are arrested in metaphase due to their failure to destroy Pds1), the effect of the artificial cleavage of Scc1p on its removal from chromosomes can be assayed (as measured by its presence in sedimented chromosomal DNA fractions). Whether or not this is sufficient for sister chromatid separation can also be examined microscopically, using the CenV-GFP system (Ciosk et al., 1998; see Example 3). These experiments allow to determine whether the rest of mitosis can proceed under these conditions in the absence of separin function (note that separin is inactive in cdc20-3 mutants at the restrictive temperature due to the presence of its inhibitor Pds1). If the foreign protease triggers Scc1p's dissociation from chromatids under these circumstances and sister chromatids segregate to opposite poles of the yeast cell, it can be concluded that cleavage of Scc1 is the sole function of

separin needed for sister chromatid segregation. If however, sister chromatids fail to segregate to opposite poles of the cell despite the variant Scc1p having been removed from chromatin, then it is concluded that separin has one or more functions besides cleavage of Scc1p. A clue as to these functions can be obtained from the phenotype of these cells and this can be used to identify other potential substrates for Esp1.

The findings of the experiments of the present invention have shed the first key insight into the molecular mechanism by which eukaryotic cells separate sister chromatids. In view of the published literature, which contains no hints as to the mechanism by which sister chromatids are separated, the finding that separins act by conferring a proteolytic activity is highly surprising.

The identification of Esp1/separin as the protease responsible for Scc1/SCC1 cleavage and the identification of potential co-factors, is the prerequisite for designing assay methods that allow for finding compounds interfering with sister chromatid separation, which is the basis for novel therapeutic approaches.

In a first aspect, the invention relates to a method for identifying compounds that have the ability of modulating sister chromatid separation in plant or animal cells, characterized in that a protease with separin-like cysteine endopeptidase activity is incubated, in the presence of the substrate(s) for its proteolytic activity and optionally its co-factor(s), with test compounds and that the modulating effect of the test compounds on the proteolytic activity of the cysteine endopeptidase is determined.

By providing a method to identify compounds which exert their effect by directly modulating, in particular by inhibiting separin's proteolytic activity, i.e. by being protease inhibitors specific for separin, the present invention provides means for interfering with the mechanism of sister chromatid separation and thus a novel approach for inhibiting the proliferation of rapidly dividing animal cells, in particular tumor cells.

In the following, if not otherwise stated, the term "separin" is used as a synonym for any cysteine endopeptidase with separin-like activity, including the yeast homolog Esp1. Similarly, the term "SCC1" is not limited to the

human separin substrate, but is intended to encompass any homologous substrate of the cohesin-type.

In a first embodiment, for small scale applications, the assay of the type as described in Example 2 for the yeast components can be used to identify compounds that inhibit separin activity. Given the existence of Esp1 homologues in man, i.e. separin, it can be concluded that the separin activity plays an important role in triggering anaphase onset also in humans. Therefore, a separin-dependent cleavage assay using human separin and SCC1 instead of yeast components can be established using the principles outlined in the experiments for yeast components. Such an assay comprises, as its essential features, incubating a crude preparation of chromatin with a preparation containing a separin activity and determining SCC1 cleavage products in the presence or absence of a test substance.

In general, when setting up a screening assay, it may be useful to first perform it with yeast constituents as assay components and subsequently further develop it stepwise using the protease and/or substrate from intermediate organisms, e.g. from S. pombe or Xenopus laevis, and finally equivalent human substrates. For example, the S. pombe homologue of Scc1 (called Rad21) contains two sequences which are similar to the two known cleavage sites in Scc1, and Rad21 derived sequences may therefore be used to generate a substrate for S. pombe Esp1 (called Cut1). This process of advancing to higher organisms can be applied stepwise until a human system is attained. The cleavage site of any new substrate for human separin can be determined by purifying the cleavage product and determining the N-terminal sequences by Edman degradation as described above.

In a preferred embodiment, the method of the invention is performed on a high-throughput scale. For this embodiment the major assay components, in particular separin, are employed in recombinant form.

Depending on the desired application of the separin-inhibitor to be identified, the assay components employed may vary in terms of the species that they are derived from. In view of therapeutical applications in animals or humans, the assay components are preferably of mammalian or

human origin, in case of intended agricultural applications, the assay components are derived from plants.

Separin can be produced recombinantly according to standard methods, e.g in yeast or insect cells or in other suitable host cells, based on the sequence information in the literature or in data bases. The obtained protein can be purified by conventional biochemical fractionation from yeast cells over-producing separin or by tagging the over-produced protein with polypeptide sequences which have a special affinity for a defined ligand (affinity purification). For example, separin can be purified on nickel-agarose columns if it has been tagged with multiple histidine residues, whereas it can be purified on glutathione-agarose columns if it has been tagged with GST. Such affinity purification involves the cleavage of separin from its tag using site specific proteases or self cleaving inteins. The thus obtained recombinant protein can then be used to determine, according to known methods for assaying proteolytic activity, whether separin alone is capable of cleaving Scc1p or peptide substrates derived from it. In case that separin is alone capable of cleaving a SCC1 or a SCC1-derived peptide, an assay based on, preferably recombinant, separin as the protease and its substrate SCC1 can easily be adapted to a high throughput format by methods that are standard for other defined proteases, as described below.

The protease substrates useful in the assay may be those equivalent to or mimicking the naturally occuring substrates, e.g. crude chromatin preparations, SCC1, preferably recombinantly produced, or an SCC1 peptide that contains the proteolytic cleavage site.

Based on information about the sequence specificity of the separin proteolytic cleavage site in yeast and in man, other potential substrates for the protease can be found in other organisms, including humans, which also allows for the design of peptides derived from these substrates, which are useful as substrates in the screening assay of the invention.

In a preferred embodiment, the substrate is a peptide containing the cleavage site of the naturally occuring substrate. The sequence specificity of the proteolytic cleavage can be determined by testing a variety of different peptides. The peptide may be of natural origin, i.e. derived from

the natural SCC1, or a variant. An example for a natural peptide ist the human SCC1 peptide as set forth SEQ:ID:NO:1, or a fragment thereof that contains the separin cleavage site. Variants can be generated either by synthesising variant peptides or by mutating DNA sequences from genes encoding cohesion proteins. More specifically, other substrates for separin can be identified by searching for small DNA fragments from the yeast genome or an oligonucleotide library that can replace the normal Scc1 cleavage sites. Oligonucleotides may be inserted into a SCC1 gene (lacking both natural cleavage sites) under control of the GAL promoter on centromeric plamid. Yeast cells may be transformed with a library of such constructs and only plasmids whose modified Scc1 protein can be cleaved by the separin activity will permit growth in the presence of galactose. The peptides encoded by the positive constructs are useful as substrates for separin in the screeing assay of the invention.

With regard to the substrate, e.g. the SCC1 protein or a peptide fragment thereof, care needs to be taken that the substrate is efficiently cleaved. It has to be considered, in particular when using the yeast homolog of SCC1, that efficient cleavage appears to occur only when the substrate is in its phosphorylated state, as it is present in mitosis. Therefore, when designing a peptide substrate or when producing SCC1 recombinantly, it has to be tested whether the substrate is efficiently cleaved by separin. In case of the recombinant protein, it can be obtained in its phosphorylated form by producing it in infected insect cells that are treated with a phosphatase inhibitor, e.g. okadaic acid. This method is exemplified, for the yeast Scc1 protein, in Example 5 (method section e) and can, if necessary for other SCC1 molecules, be adapted for these molecules.

In the case that separin does not act by itself, but in cooperation with co-factors, instead of incubating SCC1 (or peptide substrates) with separin alone, they can be incubated with a mixture of separin and its co-factors. All components can be produced and purified according to standard methods as outlined above for separin.

For the high throughput format, the screening methods of the invention to identify separin inhibitors, are carried out according to assay methods known in the art for identifying protease inhibitors. Such assays are based on the detection of the cleavage products of the substrate. To achieve this,

an SCC1 peptide or protein substrate that contains cleavage sites for the separin protease is derivatized with a detectable label, e.g a radioactive or a fluorescent label. Upon cleavage of the substrate by the protease, the cleavage product can be measured. If a test substance is an inhibitor of the protease, there will be, depending on the detection system and depending on whether the test substance has an inhibiting or an activating effect, a decrease or an increase in the detectable signal.

In the high-throughput format, compounds with a modulationg effect on separin or a separin-like cysteine endopeptidase can be identified by screening test substances from compound libraries according to known assay principles, e.g. in an automated system on microtiter plates.

Recently, various assay methods for identifying protease inhibitors have been described that are amenable to automation in a high-throughput format, e.g. the radiometric method described by Cerretani et al., 1999, for hepatitis C virus NS3 protease, the method based on fluorescence quenching described by Ambrose et al., 1998, or by Taliani et al., 1996, the microtiter colorimetric assay fot the HIV-1 protease described by Stebbins and Debouck, 1997, the fluorescence polarization assay described by Levine et al., 1997 (reviewed by Jolley, 1996), the method using immobilized peptide substrates described by Singh et al., 1996, the assay used for studying the inhibition of cathepsin G, using biotinylated and cysteine-modified peptides described by Brown et al., 1994. A further example for a suitable assay is based on the phenomenonon of fluorescence resonance energy transfer (FRET), as described by Gershkovich et al., 1996 or by Matayoshi et al., 1990. Additional examples for assays that may be used in the present invention for a high-throughput screening method to identify inhibitors of separin activity were described by Gray et al., 1994, Murray et al., 1993, Sarubbi et al., 1991.

Fluorescent or radioactive labels and the other reagents for carrying out the enzymatic reaction on a high-throughput scale are commercially available and can be employed according to supplier's instructions (e.g. Molecular Probes, Wallac). The specific assay design depends on various parameters, e.g. on the size of the substrate used. In the the case of using a short peptide, the fluorescence quenching or the fluorescence resonance nergy transfer methods are examples for suitable assay technologies.

The fluorescence quenching (Resonance Energy Transfer „RET") assay relies on synthetic substrates which are capable of direct, continuous signal generation that is proportional to the extent of substrate hydrolysis. The substrate peptide carries a fluorescent donor near one end and an acceptor near the other end. The fluorescence of the substrate is initially quenched by intramolecular RET between donor and acceptor. Upon cleavage of the substrate by the protease the cleavage products are released from RET quenching and the a fluorescence proportional to the amount of cleaved substrate can be detected.

An assay of this type may be carried out as follows: the solution of the labeled substrate (e.g. the peptide labeled with 4-[[4'-(dimethylamino)phenyl]azo]benzoic acid (DABCYL) at the one end and with 5-[(2'-aminoethyl)amino]naphtalenesulfonic acid (EDANS) at the other end or labeled with benzyloxycarbonyl at the one end and with 4-aminomethylcoumarin at the other end) in assay buffer is pipetted into each well of black 96-well microtiter plates. After addition of the test substances in the defined concentration, the separin solution is added to the wells. After incubation under conditions and for a period of time sufficient for the proteolytic cleavage reaction, e.g. for 1 hour at room temperature, the fluorescence is measured in a fluorometer at the excitation wavelength, e.g. at 340 nm, and at the emission wavelength, e.g. at 485 nm.

In the case of using the FRET assay, the following commercially availabe labeling pairs are suitable for the method of the invention: Europium (Eu) and Allophycocyanin (APC), Eu and Cy5, Eu and PE (Wallac, Turku, Finland).

The compounds identified in the above methods, which are also subject of the invention, have the ability to interfere with sister chromatid separation by modulating the proteolytic activity of a separin-like cysteine endopeptidase.

In a preferred embodiment, the compounds of the invention are inhibitors of a separin-like cysteine endopeptidase.

Preferably, the compounds are specific inhibitors of separin.

The present invention also relates to compounds which act as inhibitors of separin for use in human therapy, in particular cancer therapy.

In a further aspect, the invention relates to a pharmaceutical composition which contains, as the active ingredient, one or more compounds which interfere with or modulate sister chromatid separation by inhibiting the proteolytic activity of separin.

The present invention also encompasses inhibitors of any protease that is recognized to be a separin-like protease because of its sequence similarity to separins; i.e all proteases in which amino acid sequences surrounding the catalytic dyad are more similar to separins than to any class of protease currently known.

In a preferred embodiment, the invention comprises pharmaceutically active compounds and their use in therapy, which are small chemical molecules that have been identified as separin inhibitors in the screening method of the invention.

Alternatively, the separin inhibitors may be biological molecules, e.g. peptides or peptide-derived molecules like pepidomimetics.

Proteases from the caspase family, to which separin is likely to belong, have been shown to be good targets for irreversible binding and inhibition by peptide derived inhibitors (Nicholson et al., 1995; Faleiro et al., 1997). In principle, the approach described for the caspase inhibitors, which act as "recognition site peptides" by being modified to contain an aldehyde, halogenomethyl or acyloxymethyl group at the cleavage position, can be adapted to irreversibly bind to and inhibit the active site cysteine residue in separin. Inhibitory peptide derivatives of this type can be the starting point for rational inhibitor design, e. g. derivatives of the peptide spanning the amino acid sequence at the protease recognition site in SCC1 or another separin substrate. An example for a peptide to be considered for such design is the peptide derived from human SCC1, MDDREIMREGSAFEDDDM (SEQ:ID:NO:1), which contains the separin cleavage site, or a mutation or fragment thereof. The inhibitor design can also be aided by obtaining structural information about the catalytic domain of Esp1 using x-ray crystallography. Initially the structure of the Esp1

catalytic domain can also be modelled onto the already known structures of two members of the caspase family of proteases.

The efficacy of compounds identified as separin inhibitors can be tested for *in vivo* efficacy either on yeast cells or in mammalian cells. Effective compounds should block (or at least in some way interfere with) sister chromatid separation, which can be measured, e.g. by using CenV-GFP in yeast, as described by Ciosk et al., 1998, or standard cytological techniques in mammalian cells. Effective compounds should be either cytostatic or cytotoxic. Substances whose potential for therapeutic use has been confirmed in such secondary screen can be further tested for their effect on tumor cells. To test the inhibition of tumor cell proliferation, primary human tumor cells are incubated with the compound identified in the screen and the inhibition of tumor cell proliferation is tested by conventional methods, e.g. bromo-desoxy-uridine or ^3H incorporation. Compounds that exhibit an anti-proliferative effect in these assays may be further tested in tumor animal models and used for the therapy of tumors.

Toxicity and therapeutic efficacy of the compounds identified as drug candidates by the method of the invention can be determined by standard pharmaceutical procedures, which include conducting cell culture and animal experiments to determine the IC_{50}, LD_50, the ED_{50}. The data obtained are used for determining the human dose range, which will also depend on the dosage form (tablets, capsules, aerosol sprays, ampules, etc.) and the administration route (oral, buccal, nasal, paterental or rectal). A pharmaceutical composition containing the compound as the active ingredient can be formulated in conventional manner using one or more physologically active carriers and excipients. Methods for making such formulations can be found in manuals, e.g. "Remington Pharmaceutical Sciences".

Influencing the process of sister chromatid separation may be also beneficial in preventing birth defects caused by missegration of chromosomes in human meioses. For example, since cases of human aneuploidy such as Down's syndrome may be caused by premature separation of sister chromatids (Griffin, 1996), the use of a drug that inhibits separin activity might be able to reduce precocious sister separation and thereby the incidence of aneuploidy in human fetuses.

Thus, in a further aspect, the invention relates to separin inhibitors for the prevention of birth defects caused by missegration of chromosomes in human meioses.

Separin inhibitors may also be useful in applications which aim at the deliberate polyploidisation of plant cells for crop development. In yeast, it has been shown that inhibition of separin activity prevents chromosome separation without blocking cell cycle progression and therefore gives rise to cells with increased ploidy. Inhibitors that block separin's protease activity could therefore be used to increase the ploidy of any eukaryotic cell, including all plant cells. Increasing the ploidy of plant cells is useful for 1) producing larger plants, 2) for increasing the ploidy of breeding stocks, and 3) for generating fertile hybrids.

Therefore, the present invention relates, in a further aspect, to separin inhibitors for the treatment of plant cells for increasing their ploidy.

To identify separin inhibitors that are useful for the above-mentioned agricultural purposes, the screening method of the invention can be easily adapted by employing plant components, i.e. a plant separin and a plant homolog of SCC1. Sequence homologs of plant separin and SCC1 are present in databases, e.g. of the Arabidopsis thaliana genome.

Separin inhibitors which impair sister chromatid separation may also be used in cytological analyses of chromosomes, for example, in medical diagnoses of chromosome structure.

Brief description of the figures:

Fig. 1: Chromosome association of Scc1p in G1 is Esp1-dependent

Fig. 2: In vitro assay for Scc1p cleavage and dissociation from chromatin

Fig. 3: Detection of the Scc1p cleavage product in vivo in cells passing sychronously throught the metaphase to anaphase transition

Fig. 4: Expression of a non-cleavable variant of Scc1p prevents Scc1p dissociation from chromosomes and sister chromatid separation in vivo

Fig. 5: Purified Scc1 is a substrate for the Esp1-dependent cleavage reaction

Fig. 6: Mutations in the putative catalytic dyad of the Esp1 protease domain abolish cleavage activity

Fig. 7: Separin cleavage of the cohesin Rec8 is necessary during meiotic nuclear divisions

Fig. 8. Association of human SCC1 with chromatin

Fig. 9: Human SCC1 is cleaved in mitosis

Fig. 10: Ectopically expressed SCC1-myc is incorporated into the cohesin complex and is cleaved in mitosis

Fig.11: Human SCC1 is not cleaved in human cells arrested in metaphase by nocodazole treatment

Fig. 12: Human SCC1 is cleaved in anaphase

Fig. 13. Human SCC1-myc is cleaved in vitro

Fig. 14: Mapping of the N-terminal cleavage site of human SCC1

If not otherwise stated, the following materials and methods were used in the experiments of the present invention

a) Yeast plasmids and strains:

The Scc1p coding sequence (Saccharomyces Genome Database YDL003W) was cloned under control of the *GAL1-10* promoter in a YIplac128 derived vector (Gietz and Sugino, 1988), and under its own promoter into YCplac111 (Gietz and Sugino, 1988) using the polymerase chain reaction (PCR). DNA fragments encoding multiple HA and myc epitopes were inserted into restriction sites introduced by PCR at the N- and C-termini of SCC1. Site directed mutagenesis was performed by PCR using primers containing the desired nucleotide changes. The validity of all constructs was verified by nucleotide sequencing.

All strains used were derivatives of W303 (*HML*a *HMR*a *ho ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3*). Epitope tags at the endogenous Scc1p were generated by a PCR one-step tagging method (Michaelis et al., 1997). A strain overexpressing Esp1p from the *GAL1-10* promoter was described (Ciosk et al., 1998) and crossed to a strain containing the *esp1-1* mutation (McGrew et al., 1992). A strain expressing the sole source of Cdc20p under control of the *GAL1-10* promoter was described in (Lim et al., 1998). To visualize sister chromatids a Tet repressor-GFP fusion protein is synthesized in the cells that binds to a cluster of Tet operator sequences integrated at the *URA3* locus close to the centromere of chromosome V as described in (Michaelis et al., 1997).

All meiotic yeast strains used in Example 7 are derivatives of the rapidly sporulating SK1. The Rec8 431/453 mutant (E428R R431E R453E) was generated by site-specific mutagenic PCR of a REC8 wild-type integrative plasmid. This YIplac128-derived plasmid (Gietz and Sugino, 1988) contains the REC8 gene and promoter and 3 HA epitope sequences at the C-terminus. This plasmid was integrated at the rec8::KanMX4 locus by transformation with the MluI-linearized plasmid.

The esp1-2 allele (McGrew et al., 1992) was recovered from strain K8493 using a gap repair strategy as described by Guthrie and Fink, 1991. The recovered allele was transferred into SK1 by transformation and 5-FOA counter-selection (Guthrie and Fink, 1991). The resultant temperature-sensitive strain was diploidized by transformation with plasmid c1743 containing the HO gene. For sporulation experiments, strains were first

streaked from the -80°C stock onto a YEPGlycerol plate and grown for 60 hours at 25°C. A single colony was patched onto YEPD and grown for 48 hours at 25°C. The patch of cells was innoculated into liquid YEPD and grown for 8 hours to stationary phase. The culture was washed with YEPA and grown overnight in YEPA. Cells were washed with 2% Potassium Acetate and then incubated for 14-24 hours in the same medium. Samples were taken every two hours and fixed with 70% ethanol for visualizing nuclei by DAPI staining.

b) Yeast cell growth and cell cycle experiments

Cells were grown in complete medium (Rose et al., 1990) at 25°C if not otherwise stated. Strains expressing Cdc20p, Esp1p, or Scc1p from the *GAL1-10* promoter were grown in complete medium containing 2% Raffinose as carbon source. The *GAL1-10* promoter was induced by adding 2% galactose. A G1 like arrest was achieved by adding 1 µg/ml of the pheromone alpha factor to the medium. For a metaphase arrest, 15 µg/ml nocodazole was added with 1% DMSO. Metaphase arrest due to Cdc20p depletion was obtained in cells with the sole source of Cdc20p under control of the *GAL1-10* promoter. A logarithmically growing culture in complete medium containing raffinose and galactose was filtered, washed with medium containing Raffinose only, and resuspended in the same medium. For release from the arrest 2% Galactose was added back to the culture.

c) In vitro assay for yeast Esp1p activity

A crude Triton X-100 insoluble chromatin preparation was obtained from yeast cells as described (Liang and Stillman, 1997). The pelleted chromatin was resuspended in yeast cell extracts that had been prepared similar to the supernatant fraction of the chromatin preparation. One tenth volume of an ATP regenerating system was added (50 mM HEPES/KOH pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 600 mM creatin phosphate, 1.5 mg/ml phophocreatin kinase, 1 mM DTT, 10% glycerol). Reactions were incubated for 10 min at 25°C with shaking and stopped on ice. The chromatin fraction was separated again from the supernatant by centrifugation, and resuspended in buffer EBX (Liang and Stillman, 1997). Equivalent aliquots of supernatant and chromatin pellet were analysed by

SDS-PAGE and Western blotting. Scc1-HA was detected with the anti-HA monoclonal antibody 16B12 (Boehringer Mannheim).

Since overexpression of Esp1p from the *GAL1-10* promoter is toxic to cells, extracts with overproduced Esp1p were prepared 2 h after induction with galactose of a culture pregrown in medium containing raffinose only.

d) Protein sequencing of the yeast Scc1p cleavage site

The C-terminal Scc1p cleavage fragment was isolated from cells that contained Scc1p tagged with 18 tandem myc epitopes at the C-terminus. A Cdc20 arrest/release strategy was employed to obtain cells containing a high portion of Scc1p in the cleaved form. Protein extract of 5×10^9 cells was prepared by breakage with glass beads 15 min after release from the metaphase arrest. myc-epitope tagged protein was immunoprecipitated with 20 mg anti-myc 9E11 monoclonal antibody under denaturing conditions and resolved on SDS-PAGE next to size markers. Proteins were transferred to a PVDF membrane and stained with Coomassie Brillant Blue R250. N-terminal sequencing of the band corresponding to the Scc1p cleavage fragment yielded the amino acid sequence RLGESIM (Scc1p amino acids 269 onwards).

e) Purification of yeast Scc1 expressed in baculovirus infected insect cells

The Scc1 coding sequence was cloned into the baculovirus transfer vector pFastBac1 (Gibco Life Technologies). At the C-terminus a FLAG epitope tag was added followed by a cassette containing the yeast VMA intein and a chitin binding domain (New England Biolabs). Recombinant baculoviruses were obtained following the manufacturer's instructions. HiFive insect cells (Invitrogen) were grown in monolayers to confluency and infected at an multiplicity of infection of 2 with the recombinant baculovirus. To obtain metaphase-like phosphorylation 0.1 □M Okadaic acid was added 40 hours after infection. 43 hours after infection cells were harvested. Cytoplasmic and nuclear extracts were obtained as described (Cai et al., 1996). Scc1 was purified from the combined extracts by chitin affinity chromatography according to the manufacturer's protocols, and further purified by two subsequent ion exchange chromatography steps on a MonoQ column (Amersham Pharmacia).

f) Mutations in the yeast Esp1 catalytic dyad

Esp1 was overexpressed as described in a). The conserved residues histidine 1505 and cysteine 1531 that form the putative catalytic dyad of the Esp1 protease were changed to alanine using a PCR based mutation scheme. The mutant proteins were expressed from the GAL promoter in yeast and assayed for there cleavage activity as described under c).

g) Other yeast methods

Analysis of DNA content was performed as described (Epstein and Cross, 1992) on a Becton Dickinson FACScan, chromosome spreads were as described (Michaelis et al., 1997), photo micrographs were taken with a Photometrics CCD camera mounted on a Zeiss Axiophot microscope.

In vitro translation of Pds1p was performed in reticulocyte lysate using the TNT system (Promega).

h) Human cells

HeLa cells were cultivated in DMEM supplemented with 10% FCS at 37 °C and 5% CO_2.

In some experiments HeLa cells stably expressing mouse SCC1 fused to 9 myc ecpitopes at its C-terminus were used.

i) Human cell cycle experiments

For cell cycle synchronization, a double-thymidine treatment was used. HeLa cells were first treated with 2 mM thymidine for 18 h. Subsequently, cells were washed with PBS, fresh medium was added and the cells were grown for another 8 h. Then the cells were treated again with 2 mM thymidine for 18 h, subsequently washed and incubated in fresh medium. Samples were taken at different time points. Samples were splitted and used for FACscan analysis and for immunoblotting. Cell extracts were made with glass-teflon potters in ice cold buffer containing 50 mM Tris pH 7.7, 100 mM NaCl, 20 mM b-glycerophosphate, 5 mM $MgCl_2$, 1 mM NaF, 0.1-0.2% Triton X-100, 10% Glycerol, 1 mM DTT and protease inhibitors).

In some experiments logarithmically growing HeLa cells were treated with 330 nM nocodazole dissolved in 0.1 % DMSO for 18 hours, or cells synchronized by double-thymidine treatment were released into nocodazole-containing medium for different periods of time. In control experiments 0.1 % DMSO was added without nocodazole.

For SDS-PAGE, equal amounts of total extract was loaded (usually 50 □g protein per sample). For western blotting the following antibodies were used: Monoclonal mouse-anti-separin a C-terminal fragment of human separin was expressed in pET28Vector, His-tagged protein was purified and used for immunization); rabbit-anti-SCC1 (N-terminal or C-terminal peptides of human SCC1 were coupled to KLH and used for immunization); rabbit anti-securin (human securin was expressed in pTrcHis2 vector, His/myc-tagged protein was purified and used for immunization). All antibodies were affinity purified. CDC27, CDC20 and proteasome antibodies have been described (Gieffers et al., 1999). Mouse-anti-Cyclin B1 (#SC-245) were from Santa Cruz Biotechnology, USA. Rabbit-anti-Cyclin A (#06-138) and rabbit-anti -phopho-Histone H3 (#06-570) were from Upstate Biotechnology, USA. Rabbit-anti-myc-epitope antibodies (CM-100) were from Gramsch, Germany.

j) In vitro cleavage of SCC1-myc

Whole cell extract (containing 250 µg protein) from nocodazole-arrested HeLa cells ectopically expressing mouse SCC1-myc was separated by centrifugation into chromatin and supernatant fractions. Either 12.5 ul of the supernatant fraction or the chromatin pellet (resuspended in 5 ul of buffer containing 0.005 % Triton X100, 20 mM Hepes pH 7.7, 20 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$) were incubated with 25 µl of either interphase or mitotic Xenopus egg extract at room temperature. At different time points samples were taken, separated by SDS-PAGE and analyzed by immunoblotting with anti-myc antibodies. Xenopus extracts were prepared as described (Murray, 1991).

k) Mapping of the N-terminal cleavage site of human SCC1

For generating truncated versions of the human SCC1 cDNA, polymerase chain reactions (PCRs) were used. For N-terminal deletions different 5'-

primers containing T7 promotor regions, a start codon and appropriate SCC1 sequences were used. For C-terminal deletions different 3'-primers with appropriate SCC1 sequences and a stop-codon were used. The obtained PCR fragments were transribed and translated in the presence of ^{35}S-methionine in reticulocyte lysate in vitro (TNT system, Promega). The in vitro translated products were separated by SDS-PAGE and immunoblotted with C- or N-terminal specific SCC1 antibodies.

Example 1

Chromosome association of yeast Scc1p in G1 is Esp1-dependent

A) Cells, wild type for *ESP1* or containing the *esp1-1* mutation, with an unmodified endogenous copy of Scc1 and a second myc-tagged copy under the control of the *GAL* promoter were arrested with the mating pheromone alpha factor for 120 min. All cells had then arrested in the G1 phase of the cell cycle (time point 0 of the experiment). The FACScan profile of the DNA content is shown, demonstrating that all cells stayed arrested during the following 120 min time course of the experiment. Scc1myc was induced for 60 min by adding 2% galactose, then cells were transferred to medium containing 2% glucose to repress Scc1myc expression (Fig. 1A).

B) Expression of Scc1myc was seen by whole cell in situ hybridization (open circles), and chromosome binding of Scc1myc was observed using chromosome spreads (filled squares). The percentage of cells positive for Scc1myc expression and that had Scc1 myc bound to chromosomes is shown in the graphs (Fig. 1B).

Example 2

In vitro assay for yeast Scc1p cleavage and dissociation from chromatin

Chromatin was prepared from a strain containing Scc1p tagged with HA epitopes that was arrested in metaphase with nocodazol. The proteins in the chromatin preparation were resolved by SDS-PAGE and Scc1-HA was detected by western blotting (Fig. 2, lane 1). This chromatin preparation

was resuspended in the indicated extracts, with or without addition of in vitro translation products as indicated. Incubation was for 10 min at 25°C, after which the chromatin was separated again from the supernatant by centrifugation. Aliquots of the supernatant fraction and the chromatin fraction of each reaction were analysed.

Example 3

Detection of the yeast Scc1p cleavage product in vivo in cells passing sychronously throught the metaphase to anaphase transition

The strain used expressed Cdc20p under the control of the GAL promoter as the only source of Cdc20p. Scc1p was tagged with HA epitopes, and sister chromatids were visualized by tetR-GFP bound to tetO sequences inserted at the centromere of chromosome V. Cells were arrested at metaphase by depleting the cells of Cdc20p in medium lacking galactose for 120 min. Then 2% galactose was added to induce Cdc20p synthesis.

A) The FACscan profile of the time course is shown in Fig. 3A.

B) Budding (Fig. 3b, filled squares) was scored, all cells arrested after 120 min with large buds and cytokinesis happend for most cells between 30 min and 45 min after induction of Cdc20p synthesis. Scc1-HA bound to chromosomes was seen on chromosome spreads (Fig. 3B, open circles) in most cells in the arrest, and Scc1-HA disappeared from chromosomes within 15 min after release. The percentage of cells with separated sister chromatids as seen as the occurrence of two separated GFP dots in one cell body is presented (Fig. 3B, filled triangles).

C) Examples of cells in the arrest at 120 min and 15 min after release. The synchronous separation of sister chromatids is visible as separating GFP dots (Fig. 3C).

D) Western blot analysis of whole cell extracts at the indicated time points. The cleavage fragment of Scc1-HA is apparent at 135 min short after the release from the metaphase block into anaphase (Fig. 3D).

Example 4

Expression of a non-cleavable variant of yeast Scc1p prevents Scc1p dissociation from chromosomes and sister chromatid separation in vivo

A) FACscan profile of the DNA content as unbudded G1 cells were released into the cell cycle either with or without the induction of the Scc1RR-DD mutant (Fig. 4A).

B) Budding index without (Fig. 4B, open squares) or with (Fig. 4B, open triangles) induction of Scc1RR-DD. Sister chromatid separation in the cells was monitored by counting the percentage of cells containing two separated GFP dots (Fig. 4B, filled squares for the control culture not expressing Scc1RR-DD, and filled triangles for the culture expressing Scc1RR-DD).

C) Scc1p chromosomse association was measured on chromosome spreads. The endogenous wild type Scc1myc is shown for the control cells (open squares) and cells expressing Scc1RR-DD (Fig. 4c, open triangles). The Scc1RR-DD was HA tagged and detected on chromosome spreads of the induced culture (Fig. 4C, filled triangles).

D) Examples of chromosome spreads of both cultures at 150 min in metaphase and at 180 min when most cells of the control culture had undergone anaphase. The DNA was stained with DAPI, Scc1myc was detected with a rabbit-anti-myc antiserum and anti-rabbit-Cy5 conjugated secondary antibody, Scc1RR-DD-HA was detected with the mouse monoclonal antibody 16B12 and anti-mouse-Cy3 conjugated secondary antibody. Sister chromatids of centromere V were visualized by the GFP dots (Fig. 4D).

Example 5

Purified yeast Scc1 is a substrate for Esp1-dependent cleavage

Fig. 5A: Purification of Scc1 from baculovirus-infected insect cells. SDS-PAGE followed by staining with Coomassie brilliant blue R250 of control HiFive whole cell extract (lane 1), whole cell extract after infection with the virus expressing Scc1 (lane 2), the eluate from the chitin affinity column (lane 3), and the pooled fraction of the second MonoQ chromatographic

step (lane 4). Scc1, purified in a similar way, but containing metaphase-like phosphorylation, is shown in lane 5.

Fig. 5B: Cleavage assay using purified Scc1. Purified Scc1 in both the unphosphorylated and the metaphase-like phosphorylated form was used as a substrate in a cleavage assay. The cell extract containing Esp1 was as in Figure 2, but was used mixed in different ratios with the control extract to obtain a titration of the Esp1 activity. Scc1 was detected by Western blotting with the anti-FLAG monoclonal antibody M2 (Sigma).

Example 6

Mutation of the catalytic dyad in yeast Esp1 abolishes its cleavage activity. Wildtype Esp1 and mutants H1505A and C1531A were overexpressed in yeast, tagged with a FLAG epitope for detection. Fig 6A: Western blot of cell extracts showing that the two mutant Esp1 proteins were expressed as stable proteins to similar levels as the wild type protein. Fig. 6B: The associated Scc1 cleavage activity was assayed as in Example 2.

Example 7

Preventing cleavage of the meiotic cohesion protein Rec8 by mutations in its cleavage sites or by an esp1 mutation inhibits meiotic nuclear divisions

Fig. 7A: Diploid yeast strains either wild type for Rec8 or expressing Rec8 with both cleavage sites mutated were sporulated. The percentage of cells containing either one nucleus, two nuclei, or three or four nuclei is depicted throughout the time course of the experiment.

Fig. 7B: A diploid yeast strain homozygous for the esp1-2 mutation was sporulated at 25°C or 35°C. The percentage of cells containing either one nucleus, two nuclei, or three or four nuclei is depicted.

Example 8

Association of human SCC1 with chromatin

Chromatin and supernatant fractions were prepared from HeLa cells that were ither growing logarithmically (log) or were arrested in metaphase with nocodazole (noc). Proteins from equivalent aliquots of these fractions

were separated by SDS-PAGE and analyzed for the presence of SCC1 by immunoblotting using radiolabeled antibodies (Fig. 8, top panel), The intensities of the SCC1 bands were quantitated (Fig. 8, bottom panel).

Example 9

Human SCC1 is cleaved in mitosis

HeLa cells were arrested at the onset of S-Phase by a double-thymidine treatment and were synchronously released into the cell cycle. Samples were taken at the indicated time points. Cells were analyzed for their DNA content by FACscan (Fig. 9A) and by SDS-PAGE and immunoblotting of whole cell extracts using the indicated antibodies (Fig. 9B). The phosphorylation-dependent electrophoretic mobility shift of the APC subunit CDC27 was used as a marker for mitotic entry. The disapperance of cyclin A was used as a marker for metaphase, and the disappearance of CDC20, securin and cyclin B as a marker for anaphase. Proteasome levels were determined as a loading control. The arrows in panels 3 and 4 of Fig. 9B from the top indicate 100 and 25 kDa bands that are recognized by antibodies that are specific for the C- and the N-terminus of SCC1, respectively.

Example 10

Ectopically expressed SCC1-myc is incorporated into the cohesin complex and is cleaved in mitosis

Fig. 10A: An extract of logarithmically growing HeLa cells stably expressing SCC1-myc was separated by 5-20% sucrose density gradient centrifugation. Proteins from each gradient fraction were analyzed by SDS-PAGE and immunoblotting using antibodies to SCC1, the myc epitope and against the human cohesin subunit SA1. The position of 9S and 14S cohesin complexes is indicated. Both endogenous SCC1 and ectopically expressed SCC1-myc are exclusively found in the fractions containing the 14S cohesin complex.

Fig. 10B: HeLa cells stably expressing SCC1-myc were arrested by double-thymidine treatment and synchronously released into the cell cycle. Samples were taken at the indicated time points and whole cell extracts

were analyzed by SDS-PAGE and immunoblotting using antibodies agaist the myc epitope and the C-terminus of SCC1. SCC1 cleavage products are indicated by arrows. Two exposures of the myc immunoblot are shown to reveal a second SCC1-myc cleavage product of higher electrophoretic mobility that can only be detected in prolonged exposures (bottom panel).

Example 11

Human SCC1 is not cleaved in human cells arrested in metaphase by nocodazole treatment

HeLa cells stably expressing SCC1-myc were synchronized by double-thymidine treatment and were released into the cell cycle in the presence of either DMSO (results shown in Fig. 11A and C) or nocodazole dissolved in DMSO (results shown in Fig. 11B and D). At indicated time points samples were taken. Cells were analyzed by FACsan (Fig. 11A and B) and by immunoblotting of whole cell extracts using the indicated antibodies (Fig. 11 C and D). Antibodies specific for the C-terminus of SCC1 were used. In addition to the antibodies used in Example 9, an antibody specific for mitotically phosphorylated histone H3 (PhosphoH3) was used as a mitotic marker.

Example 12

SCC1 is cleaved in anaphase

HeLa cells stably expressing SCC1-myc were arrested in metaphase with nocodazole and synchronously released into anaphase. Samples were taken at the indicated time points. Cells were analyzed by FACscan (Fig. 12A) and by immunoblotting of whole cell extracts using the indicated antibodies (Fig. 12B). Antibodies specific for the C-terminus of SCC1 were used.

Example 13

SCC1-myc is cleaved in vitro

Chromatin and supernatant fractions were isolated by differential centrifugation from nocodazole-arrested HeLa cells stably expressing SCC1-myc. The chromatin fraction was incubated in mitotic and interphase

Xenopus egg extracts and the supernatant fraction in mitotic Xenopus egg extract. Samples were taken at the time points indicated in Fig. 13 and analyzed by SDS-PAGE and immunoblotting using antibodies to the myc epitope. Whole cell extracts from SCC1-myc expressing HeLa cells in S-phase or in anaphase (obtained by release from a double-thymidine arrest for 6 and 11.5 hours, respectively) were analyzed side by side. Cleavage products of SCC1-myc that are specifically formed in mitotic Xenopus extrcats are indicated by arrows.

Example 14

Mapping of the N-terminal cleavage site of human SCC1

Truncated versions of the human SCC1 cDNA were generated by PCR and transcribed and translated in vitro. The ^{35}S-labeled in vitro translation products (^{35}S-IVT) were analyzed by immunoblotting with SCC1 antibodies (top panels) and by phosphorimaging (bottom panels). N-terminal deletion mutants were analyzed by immunoblotting with antibodies specific for the C-terminus of SCC1 (Fig. 14, left panels) and C-terminal deletion mutants were analyzed with antibodies specific for the N-terminus of SCC1 (Fig. 14, right panels). Extracts from SCC1-myc expressing HeLa cells in anaphase or in G1 (obtained by release from a double-thymidine arrest) were analyzed side by side. The SCC1 cleavage products detected in HeLa extracts are indicated by arrows.

References

Ambrose WP, et al., (1998), *Anal Biochem*, Oct 15, **263**(2): 150-7

Brown AM, et al., (1994), *Anal Biochem*, Feb 15, **217**(1): 139-47

Cai J, et al., (1996): Reconstitution of human replication factor C from its five subunits in baculovirus-infected insect cells. *Proc. Natl. Acad. Sci. USA* **93**, 12896-12901

Cerretani M, et al., (1999), *Anal Biochem,* Jan 15, **266**(2): 192-7

Ciosk R, Zachariae W, Michaelis C, Shevchenko A, Mann M, and Nasmyth K (1998): An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell* **93**, 1067-1076

Cohen-Fix O, Peters J-M, Kirschner MW, and Koshland D (1996): Anaphase initiation is Sacharomyces cervisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev* **10**, 3081-3093

Dougherty WG, Cary SM and Dawn Parks T (1989): Molecular Genetic Analysis of a Plant Virus Polyprotein Cleavage Site: A Model. Virology 171, 356-364

Dominguez A, Ramos-Morales F, Romero F, Rios RM, Dreyfus F, Tortolero M and Pintor-Toro JA (1998): hpttg, a human homologue of rat pttg, is overexpressed in hematopoietic neoplasms. Evidence for a transcrptional activation function of hPTTG. *Oncogene* **17**: 2187-2193

Epstein CB, and Cross FR (1992) CLB5: a novel cyclin from budding yeast with a role in S-phase. *Genes Dev.* **6**, 1695-1706

Faleiro L, Kobayashi R, Fearnhead H, and Lazebnik Y (1997): Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. *EMBO J.* **16**, 2271-2281

Funabiki H, et al., (1996): Cut2 proteolysis required for sister-chromatid separation in fission yeast. *Nature* **381**, 438-441

Gershkovich, A.A. and Kholodovych, V.V. (1996), J Biochem Biophys Meth 33, 135

Gietz RD and Sugino A (1988): New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**, 527-534

Gray NM, et al., (1994), *Anal Biochem*, **216**(1): 89-96

Guthrie, C. and Fink, G.R, editors (1991): Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology, Volume 194, Academic Press, Inc., USA

Hayden JH, Bowser SS, and Rieder CL (1990): Kinetochores capture astral microtubules during chromosome attachment to the mitotic spindle: direct visualization in live newt lung cells. *J. Cell Biol.* **3**, 1039-1045

Jolley, M.E. (1996), J Biomol Screening 1, 33

Kramer ER, Gieffers C, Hölzl G, Hengstschläger M and Peters JM (1998): Activation of the human anaphase-promoting complex by proteins of the CDC10/Fizzy family. *Current Biology* **8**: 1207-1210

Kerrebrock AW, Moore DP, Wu JS, and Orr-Weaver TL (1995): Mei-S332, a Drosophila protein required for sister chromatid cohesion, can localize to meiotic centromere regions. *Cell* **83**, 247-256

Klein F, et al., (1999): A central role for the cohesins in sister chromatid cohesion , formation of axial elements, and recombination during yeast meiosis. *Cell* **98**, 91-103

Levine LM, et al., (1997), *Anal Biochem*, Apr 5, **247**(1): 83-8

Liang C, and Stillman B (1997): Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. *Genes Dev* **11**, 3375-3386

Lim HH, Goh P-Y, and Surana U (1998): Cdc20 is essential for the cyclosome-mediated proteolysis of both Pds1 and Clb2 during M phase in budding yeast. *Curr. Biol.* **8**, 231-234

Losada A, Hirano M, and Hirano T (1998): Identification of Xenopus SMC protein complexes required for sister chromatid cohesion. *Genes Dev* **12**, 1003-1012

Matayoshi, E.D. (1990), Science 247, 954

McKay MJ, et al., (1996): Sequence conservation of the rad21 Schizosaccharomyces pompe DNA double-strand break repair gene in human and mouse. *Genomics* **36**, 305-315

McGrew JT, Goetsch L, Byers B, and Baum P (1992): Requirement for ESP1 in the nuclear division of S. cerevisiae. *Mol. Biol. Cell* **3**, 1443-1454

Merdes A, and De Mey J (1990): The mechanism of kinetochore-spindle attachment and polewards movement analyzed in PtK2 cells at the prophase-prometaphase transition. *Eur. J. Cell Biol.* **53**, 313-325

Michaelis C, Ciosk R, and Nasmyth K (1997): Cohesins: Chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91**, 35-45

Miyazaki WY, and Orr-Weaver TL (1994): Sister chromatid cohesion in mitosis and meiosis. *Annu. Rev. Genet.* **28**, 167-187

Moore DP, Page AW, Tang TTL, Kerrebrock AW, and Orr-Weaver TL (1998): The cohesion protein Mei-S322 localizes to condensed meiotic and mitotic centromeres until sister chromatids separate. *J. Cell Biol.* **140**, 1003-1012

Murray AW (1991): Cell cycle extracts. Methods In cell biology **36**, 581-605

Murray MG, et al., (1993), *Gene*, Nov 30, **134**(1):123-8

Nagase T, Seki N, Ishikawa K, Tanaka A, and Nomura N (1996): Prediction of the coding sequences of unidentified human genes. V. The coding

sequences of 40 new genes (KIAA0161-KIAA0200) deduced by analysis of cDNA clones from human cell line KG-1. *DNA Res* **3**, 17-24

Nicholson DW, et al., (1995): Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376**, 37-43

Peters J-M (1998): SCF and APC the Yin and Yan of cell cycle regulated proteolysis. *Curr. Op. Cell Biol.* **10**, 759-768

Remington's Pharmacuetical Sciences, 1980, Mack Publ. Co. Easton, PA, Osol (ed.)

Rieder CL, and Salmon ED (1998): The vertebrate cell kinetochore and its roles during mitosis. *Trends Cell. Biol.* **8**, 310-318

Rose MD, Winston F, and Hieter P (1990): Laboratory course manual for methods in Yeast Genetics (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York)

Sarubbi E, et al., (1991), *FEBS Lett*, Feb 25, **279**(2): 265-9

Singh J, et al., (1996), *Bioorg Med Chem*, **4**(5): 639-43

Stebbins J. and Debouck C., (1997), *Anal Biochem*, Jun 1, **248**(2): 246-50

Taliani M, et al., (1996), *Anal Biochem*, Aug 15, **240**(1):60-7

Uhlmann F, and Nasmyth K (1998): Cohesion between sister chromatids must be established during DNA replication. *Curr. Biol.* **8**, 1095-1101

Zou H, McGerry TJ, Bernal T, Kirschner MW (1999): Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. *Science* **285**:418-422

Claims

1. A method for identifying compounds that have the ability of modulating sister chromatid separation in plant or animal cells, characterized in that a protease with separin-like cysteine endopeptidase activity is incubated, in the presence of the substrate(s) for its proteolytic activity and optionally its co-factor(s), with test compounds and that the modulating effect of the test compounds on the proteolytic activity of the cysteine endopeptidase is determined.

2. The method of claim 1, wherein said protease is recombinant.

3. The method of claim 1 or 2, wherein said protease is human separin.

4. The method of claim 1, wherein said substrate is a protein recombinantly produced in baculovirus in the presence of a phosphatase inhibitor.

5. The method of claim 1, wherein the substrate is human SCC1 oder a fragment or variant thereof.

6. The method of claim 5, wherein the substrate is a peptide with the amino acid sequence as set forth in SEQ:ID:NO:1 or a cleavable fragment or variant thereof.

7. The method of claim 1 or 2, wherein the protease is a plant separin.

8. The method of any one of claims 1 to 7, wherein the substrate carries a label which generates a detectable signal proportional to the amount of the cleavage product of the proteolytic activity, and wherein the signal is measured in the presence and in the absence of the test compound.

9. The method of claim 8, wherein the label is fluorescent.

10. Inhibitors of a protease with separin-like cysteine endopeptidase activity for human therapy.

11. Inhibitors of human separin for human cancer therapy.

12. The use of inhibitors of a protease with separin-like cysteine endopeptidase activity for the preparation of a medicament for the treatment of cancer.

13. Inhibitors of human separin for the prevention of birth defects caused by missegration of chromosomes in meioses.

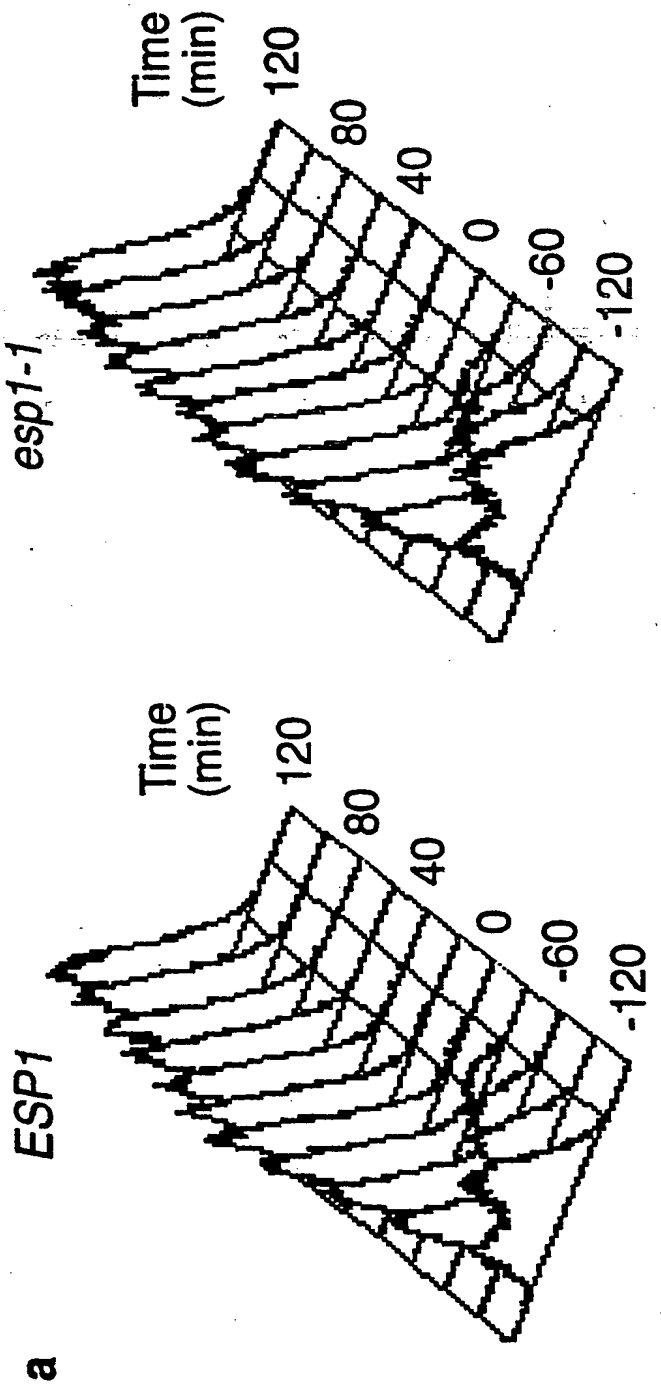
14. The use of inhibitors of a protease with separin-like cysteine endopeptidase activity for the preparation of a medicament for the prevention of birth defects caused by missegration of chromosomes in meioses.

15. Pharmaceutical composition, containing as active ingredient an inhibitor of human separin.

16. Inhibitors of plant separin for increasing the ploidy of plant cells.

1/24

Fig. 1 a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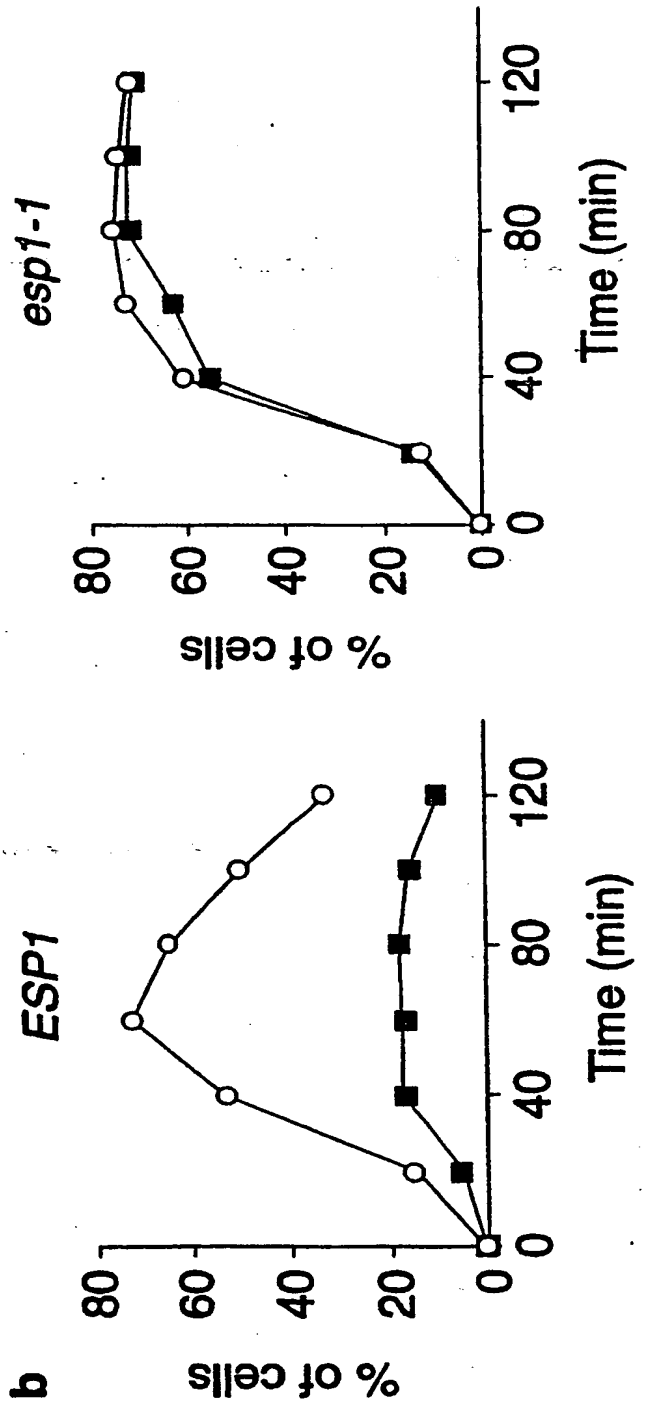
2/24

Fig. 1 b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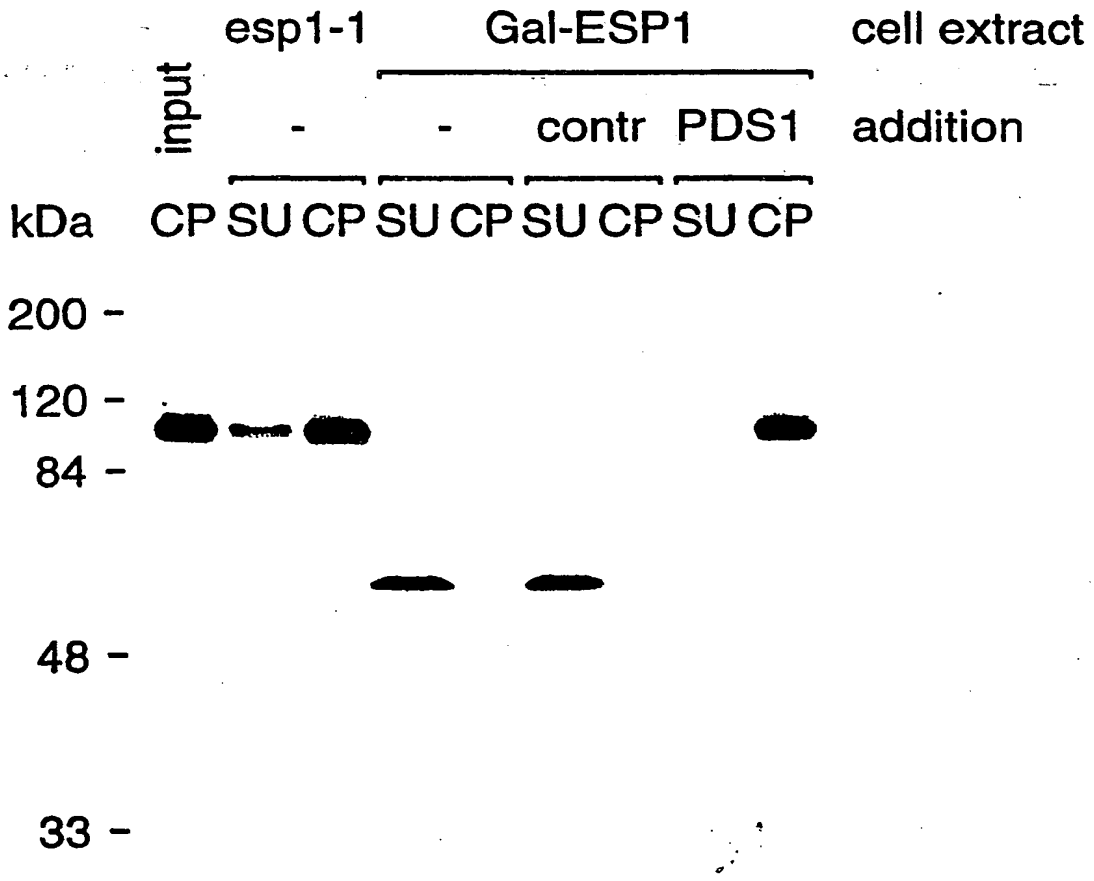
3/24
Fig. 2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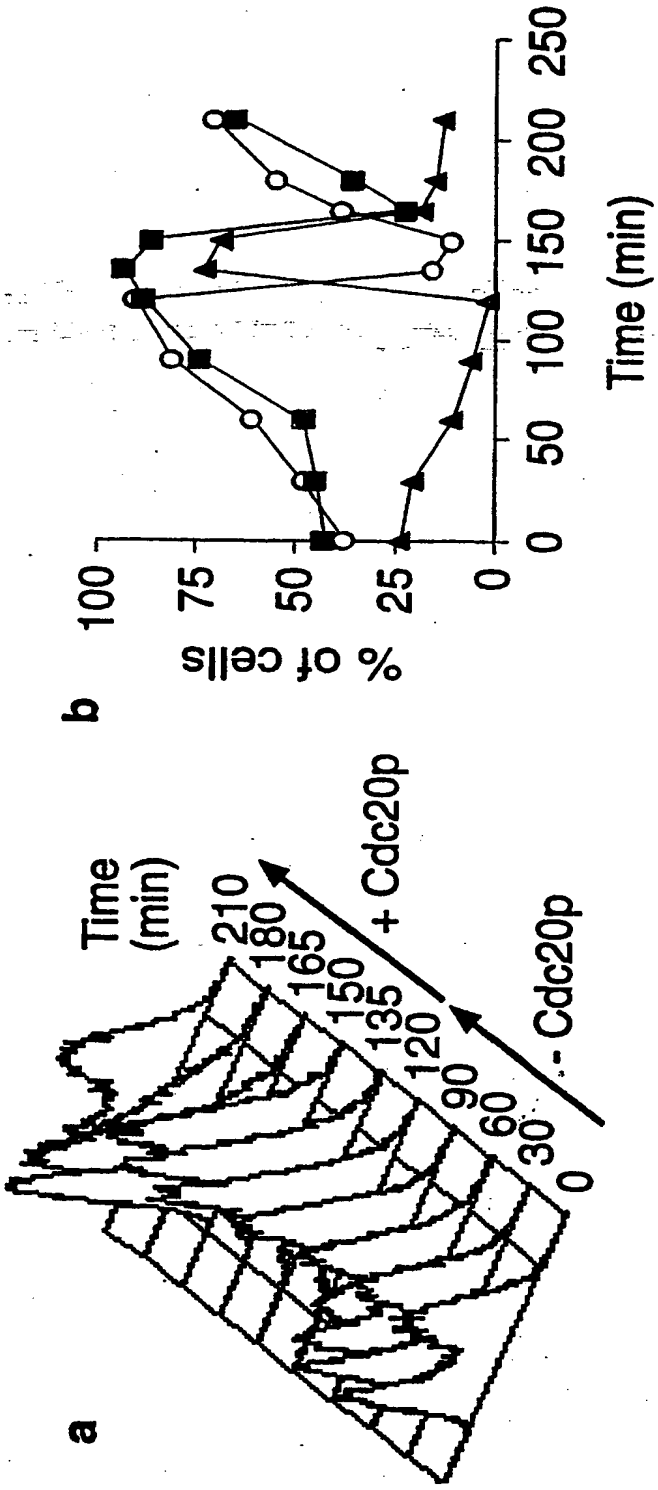
4/24

Fig. 3 a, b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5/24
Fig. 3 c, d

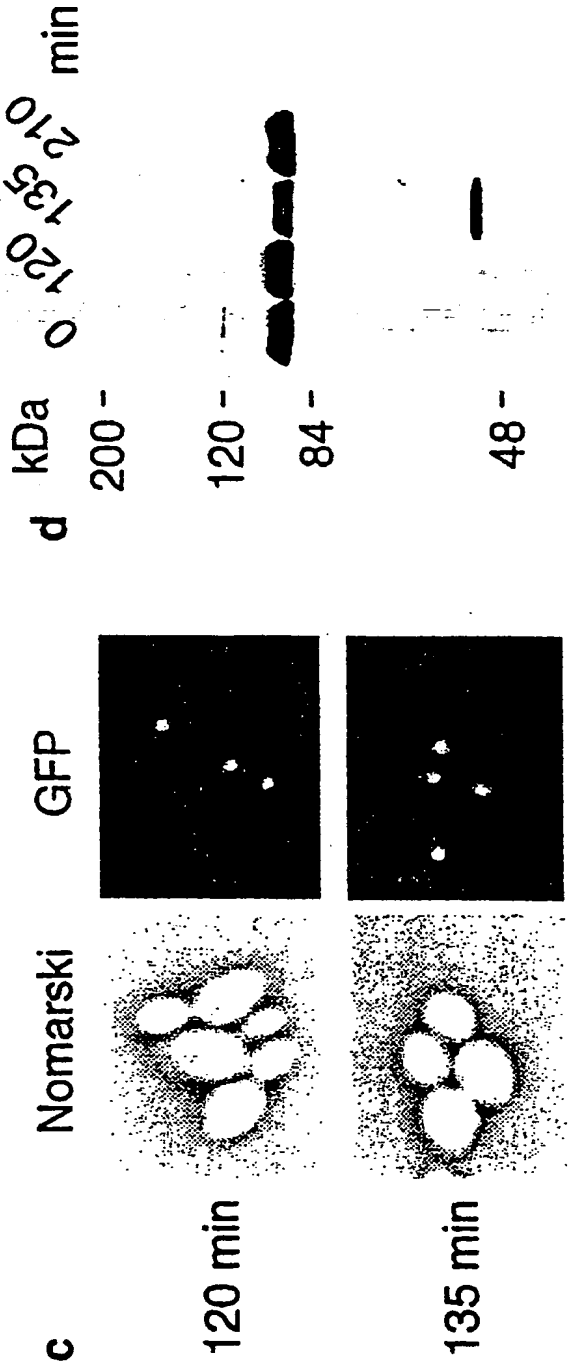
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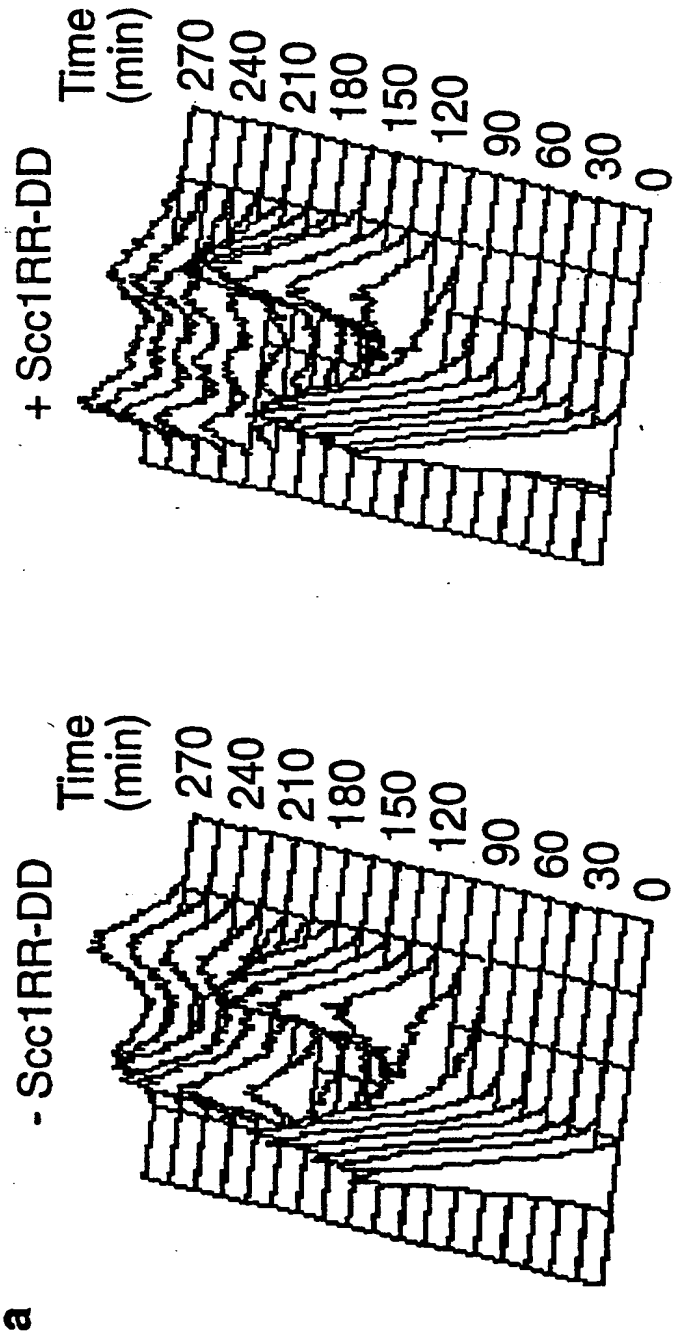
Fig. 4 a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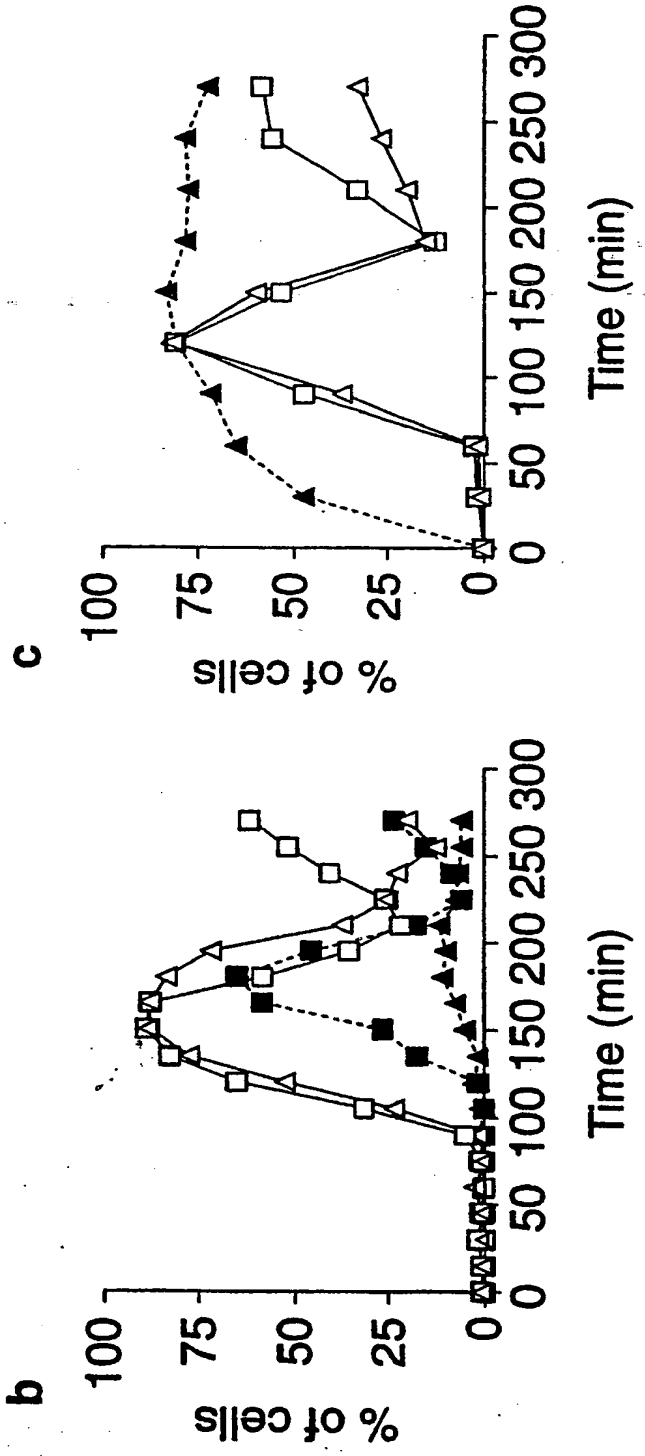
a

7/24

Fig. 4 b, c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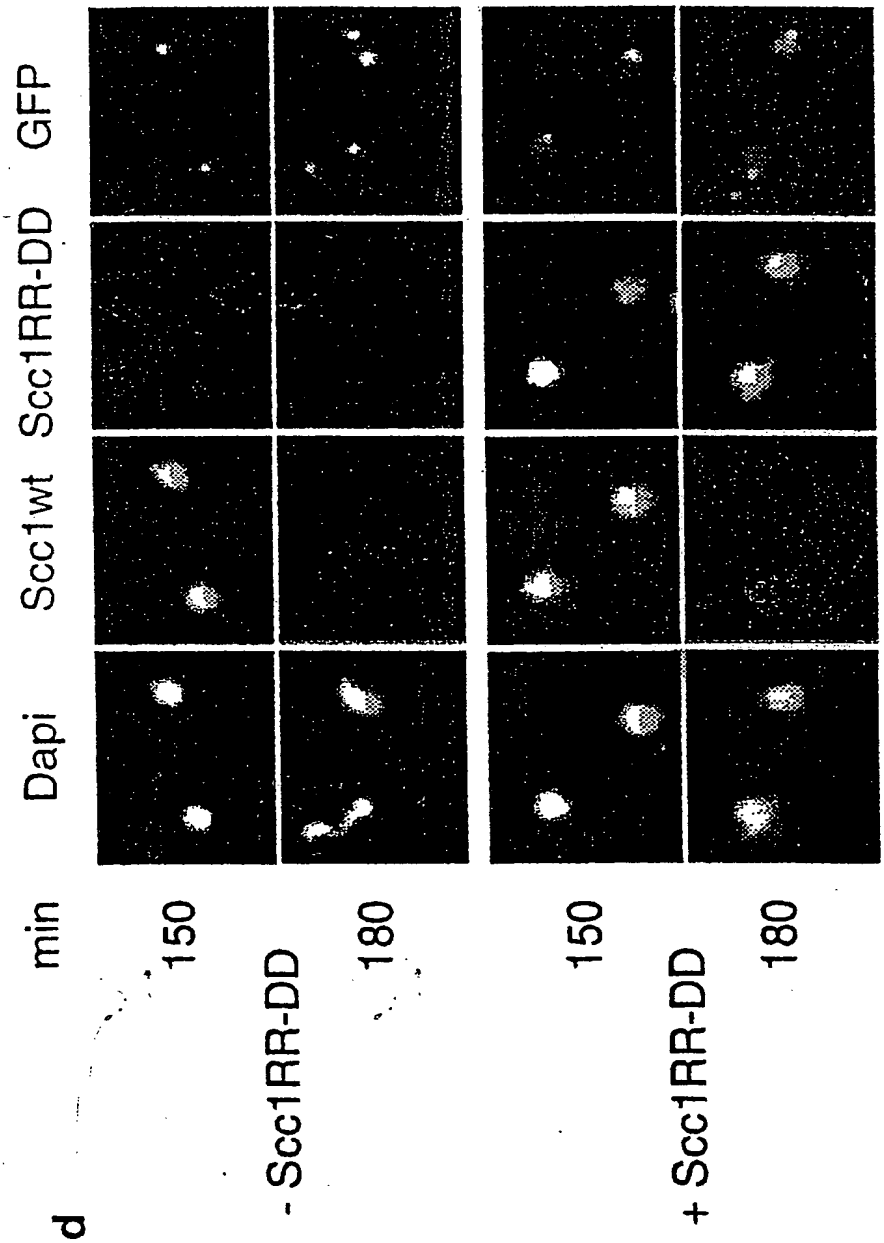
Time (min)

8/24

Fig. 4 d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9/24
Fig. 5

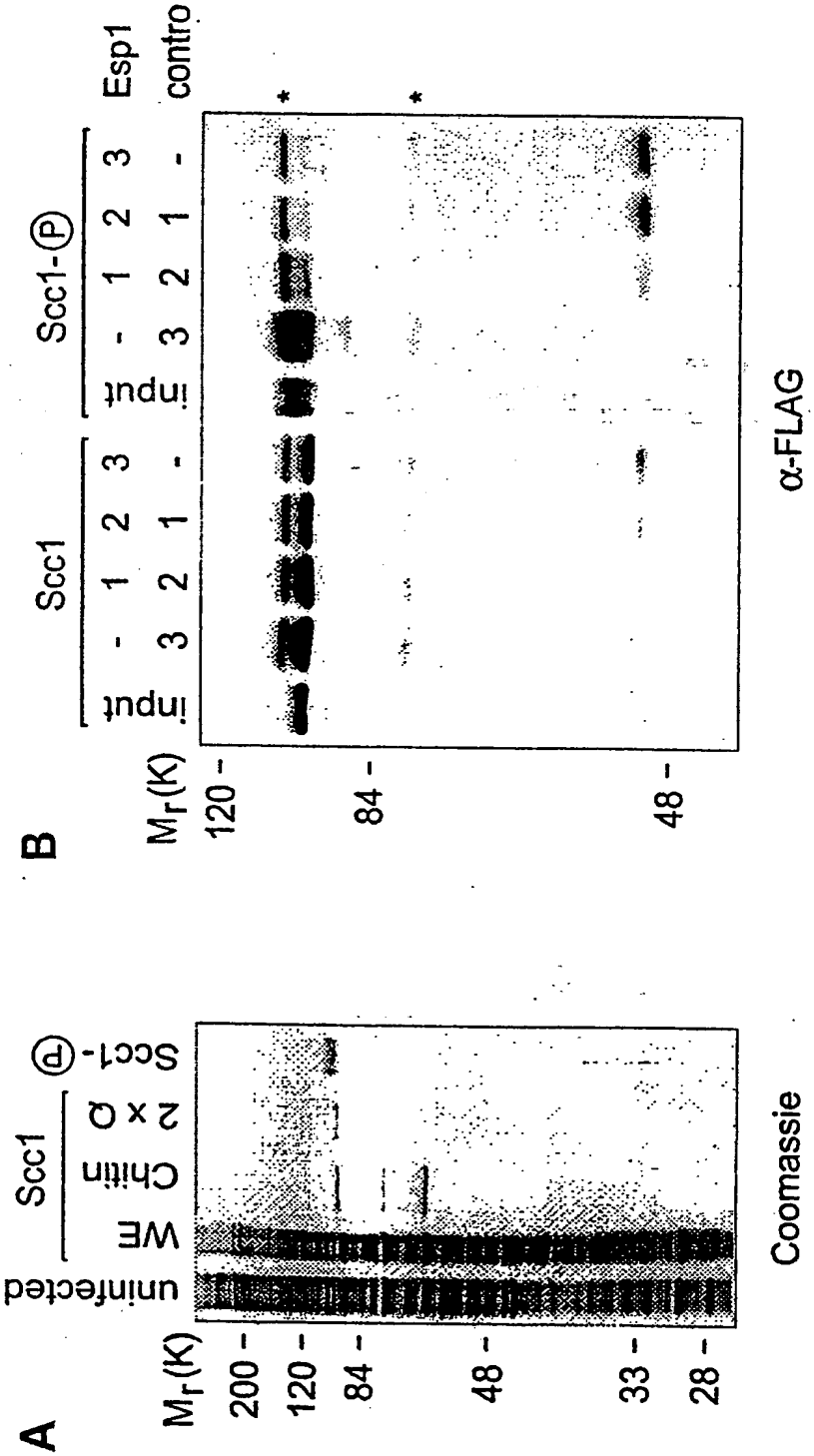
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Fig. 6

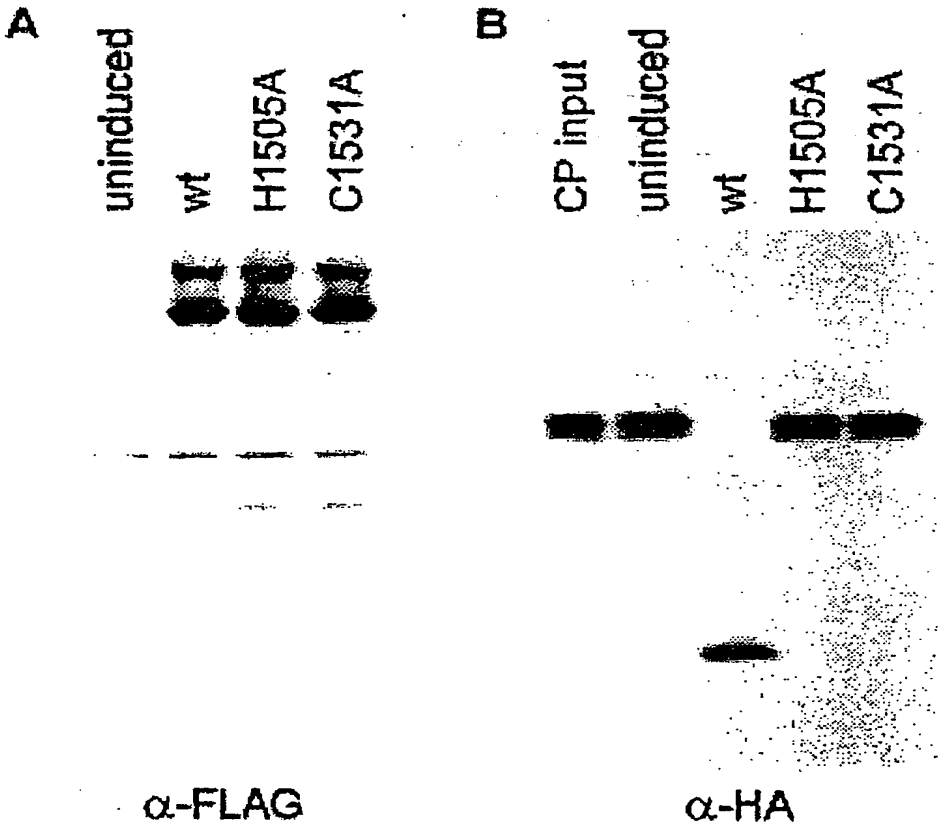
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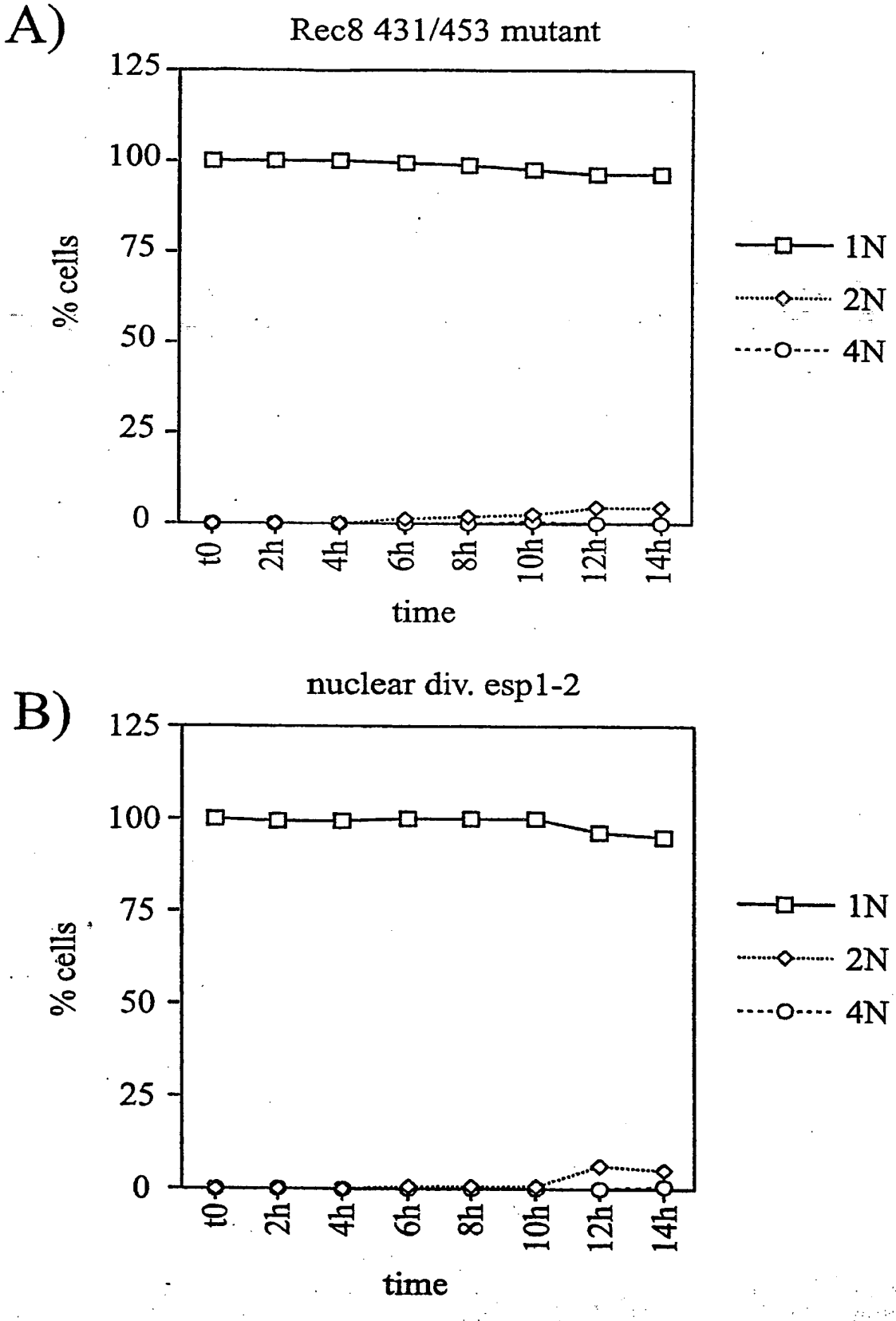
Fig. 7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Rec8 431/453 mutant
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75
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25
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% cells
t0
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4h
6h
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10h
12h
14h
time
1N
2N
4N
B)
nuclear div. esp1-2
125
100
75
50
25
0
% cells
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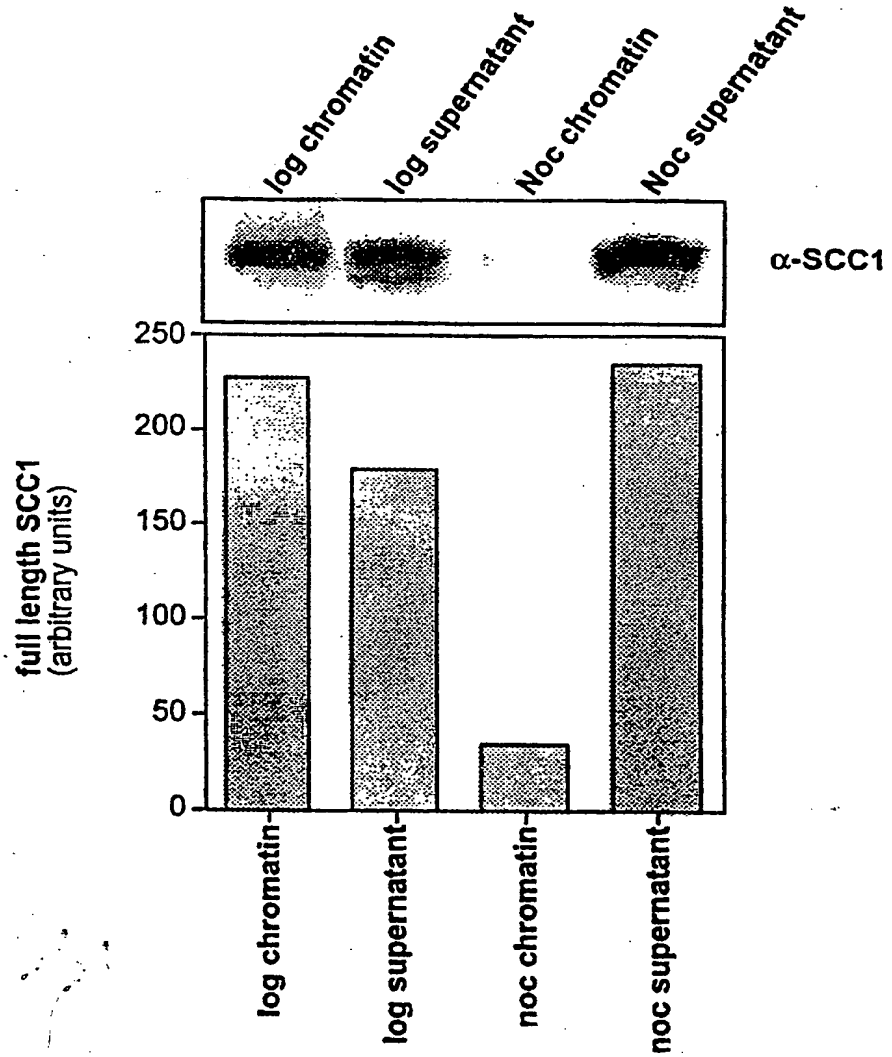
8h
10h
12h
14h
time
1N
2N
4N

12/24
Fig. 8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Noc chromatin
Noc supernatant
α-SCC1
full length SCC1
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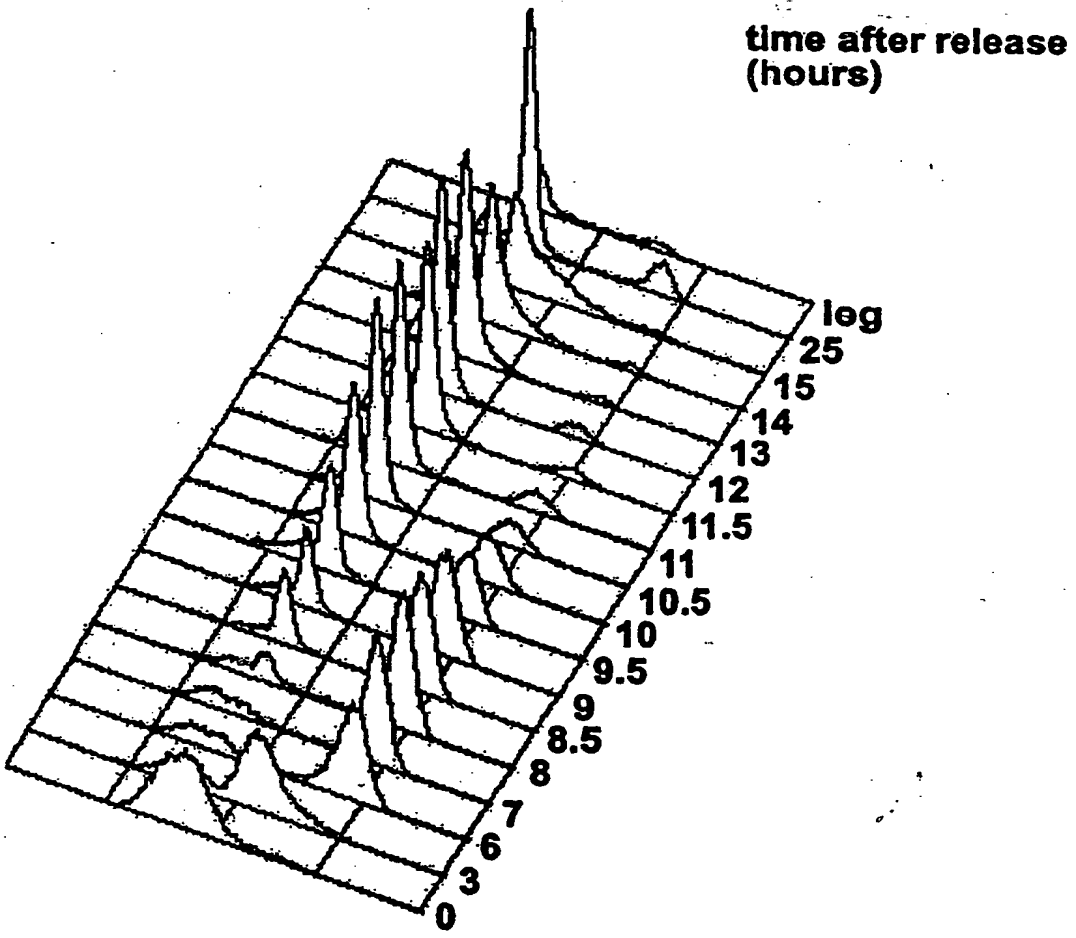
150
100
50
0
log chromatin
log supernatant
noc chromatin
noc supernatant

13/24

Fig. 9 a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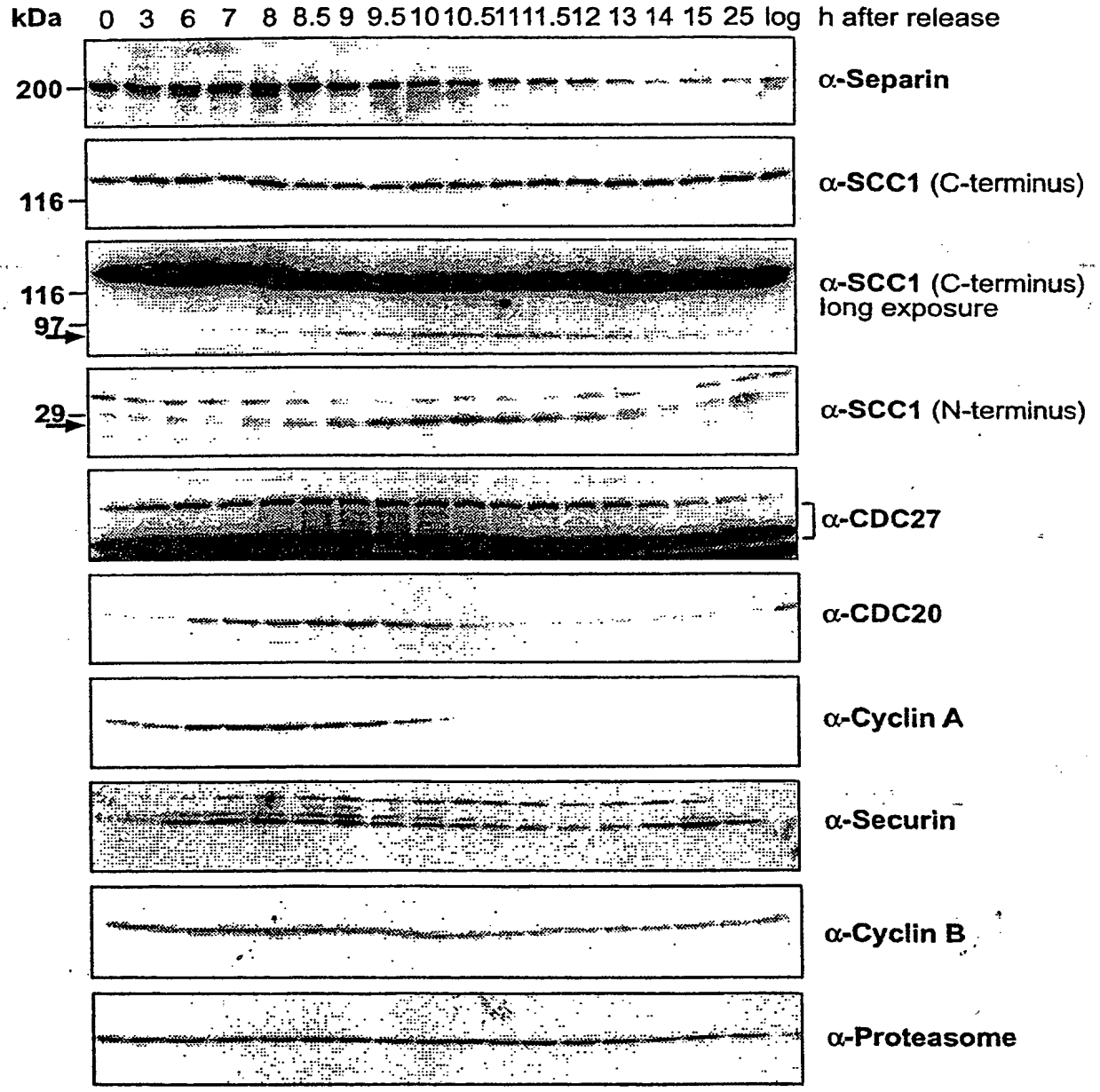
14/24
Fig. 9 b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h after release
200
α-Separin
116
α-SCC1 (C-terminus)
116
97
α-SCC1 (C-terminus)
long exposure
29
α-SCC1 (N-terminus)
α-CDC27
α-CDC20
α-Cyclin A
α-Securin
α-Cyclin B
α-Proteasome

15/24

Fig. 10 a

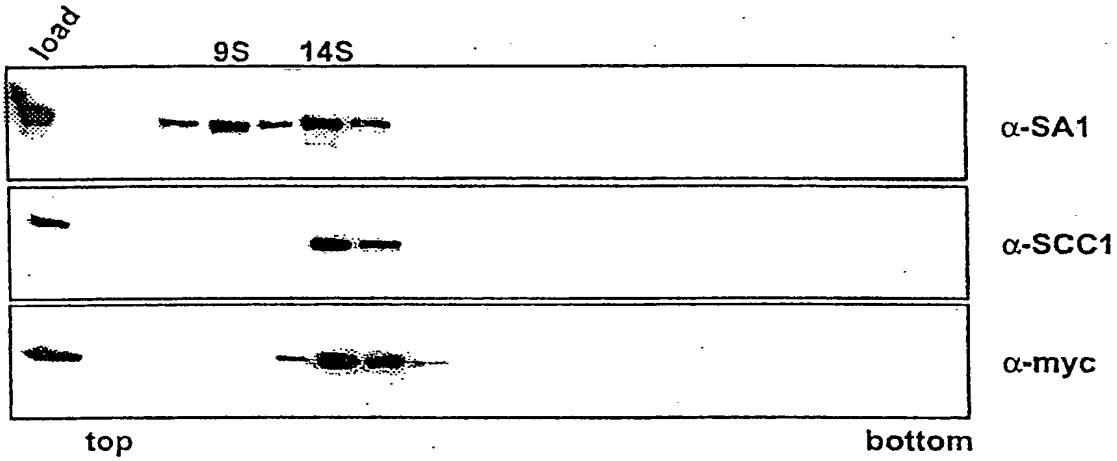

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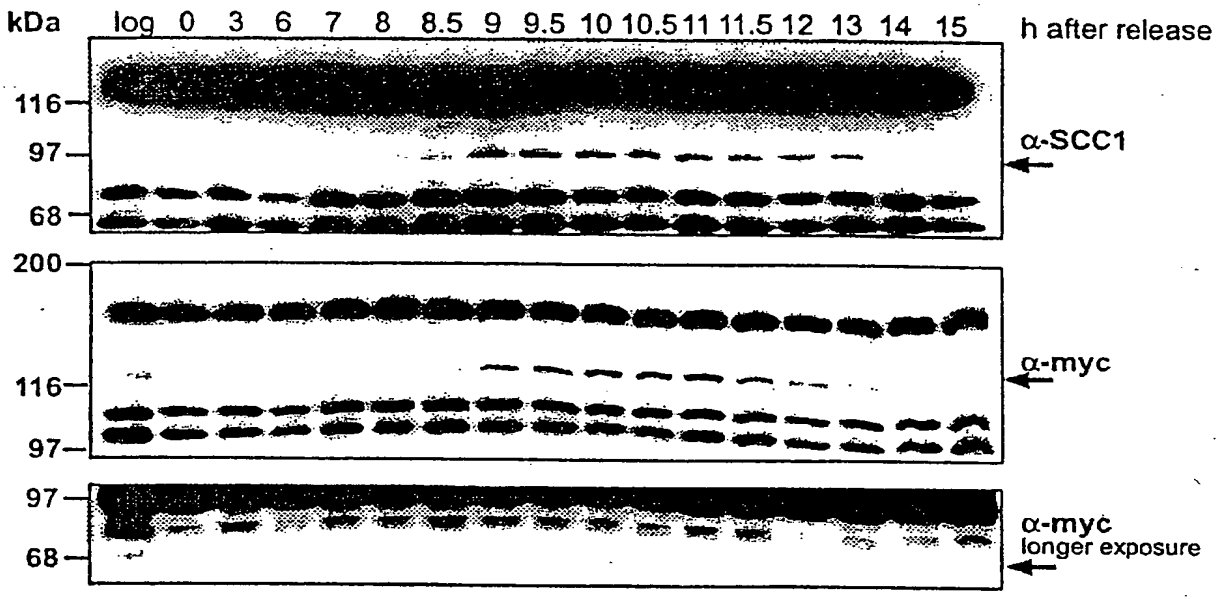
Fig. 10 b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h after release
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97
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α-SCC1
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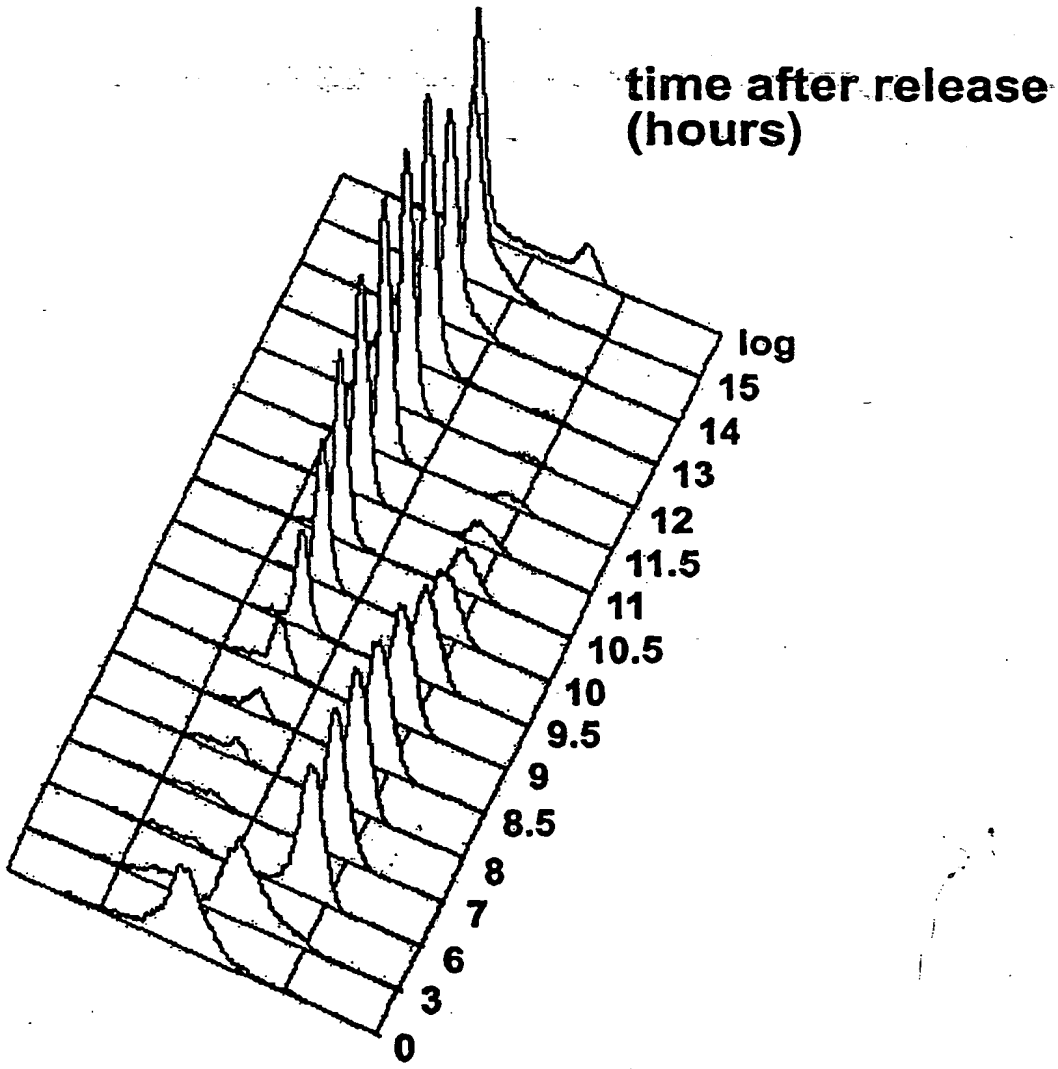
116
97
α-myc
97
68
α-myc
longer exposure

17/24

Fig. 11 a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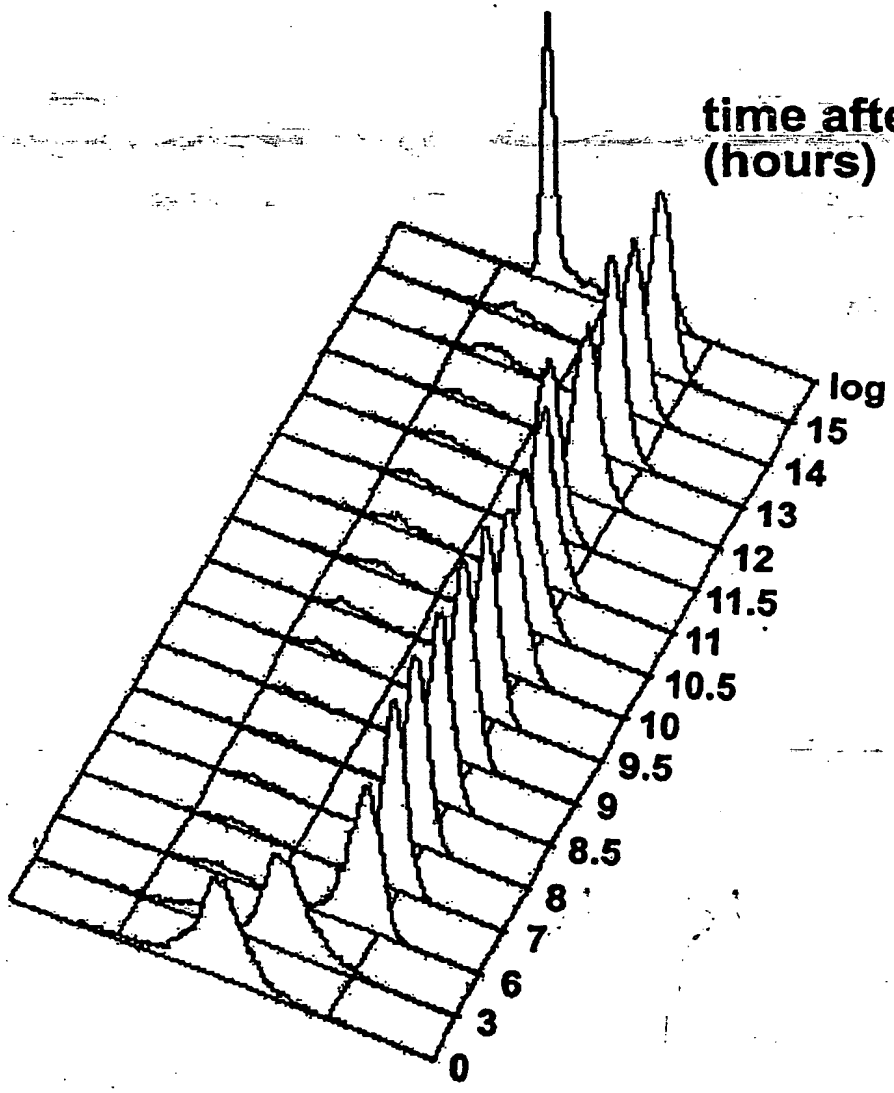
3
0

18/24
Fig. 11 b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(hours)
log
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14
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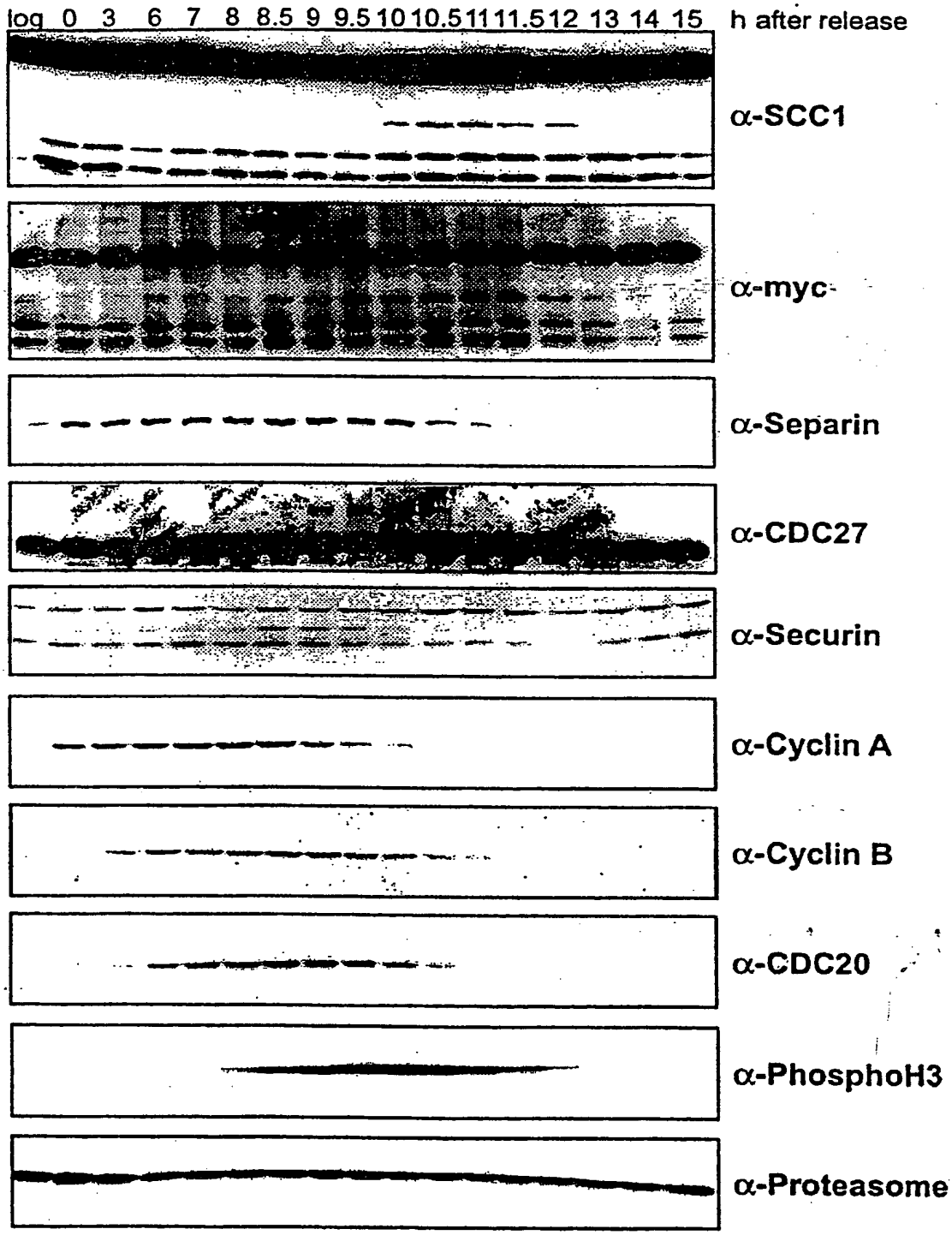
6
3
0

19/24

Fig. 11 c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α-myc
α-Separin
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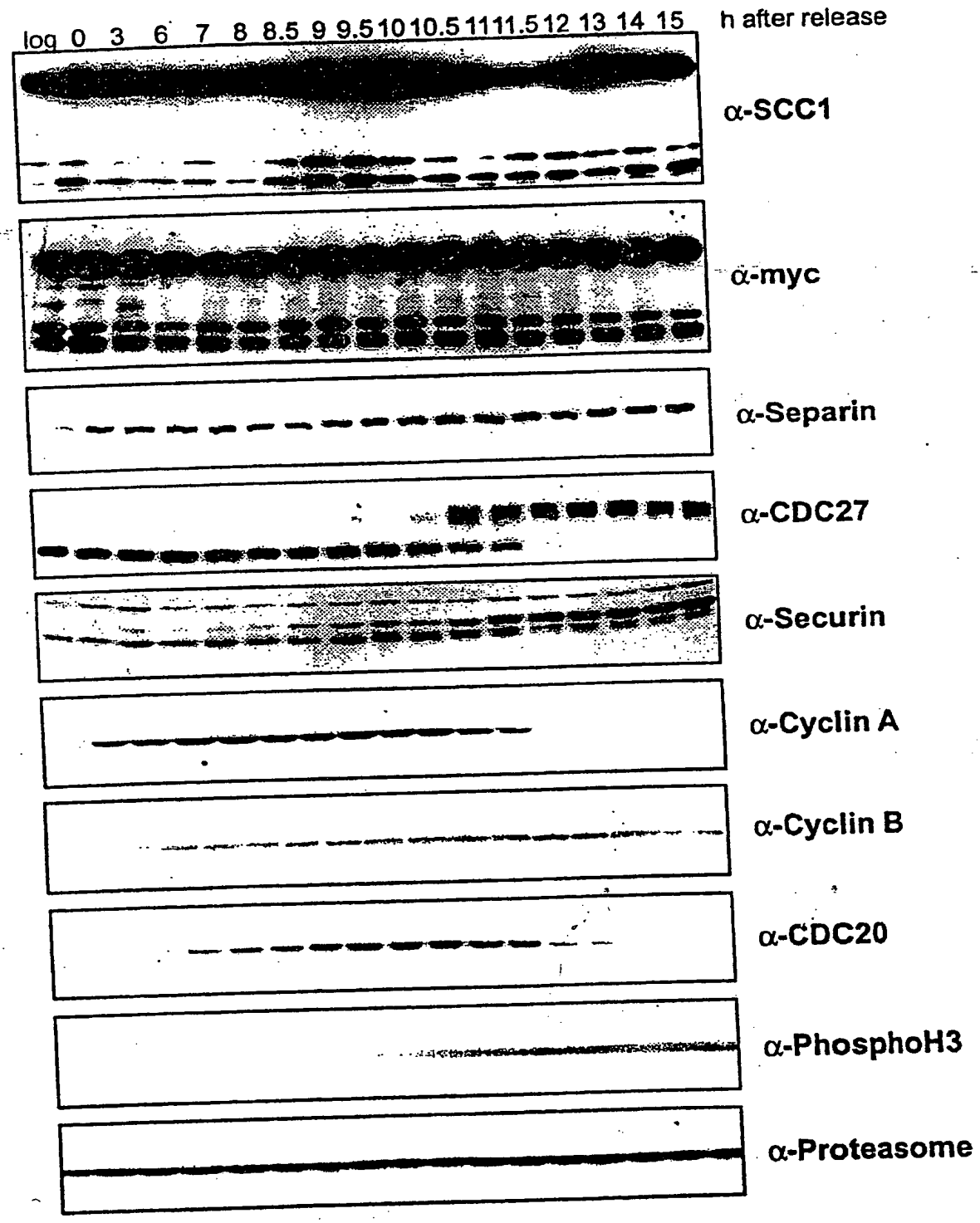
α-PhosphoH3
α-Proteasome

20/24

Fig. 11 d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α-Separin
α-CDC27
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α-Cyclin B
α-CDC20
α-PhosphoH3
α-Proteasome

21/24
Fig. 12 a

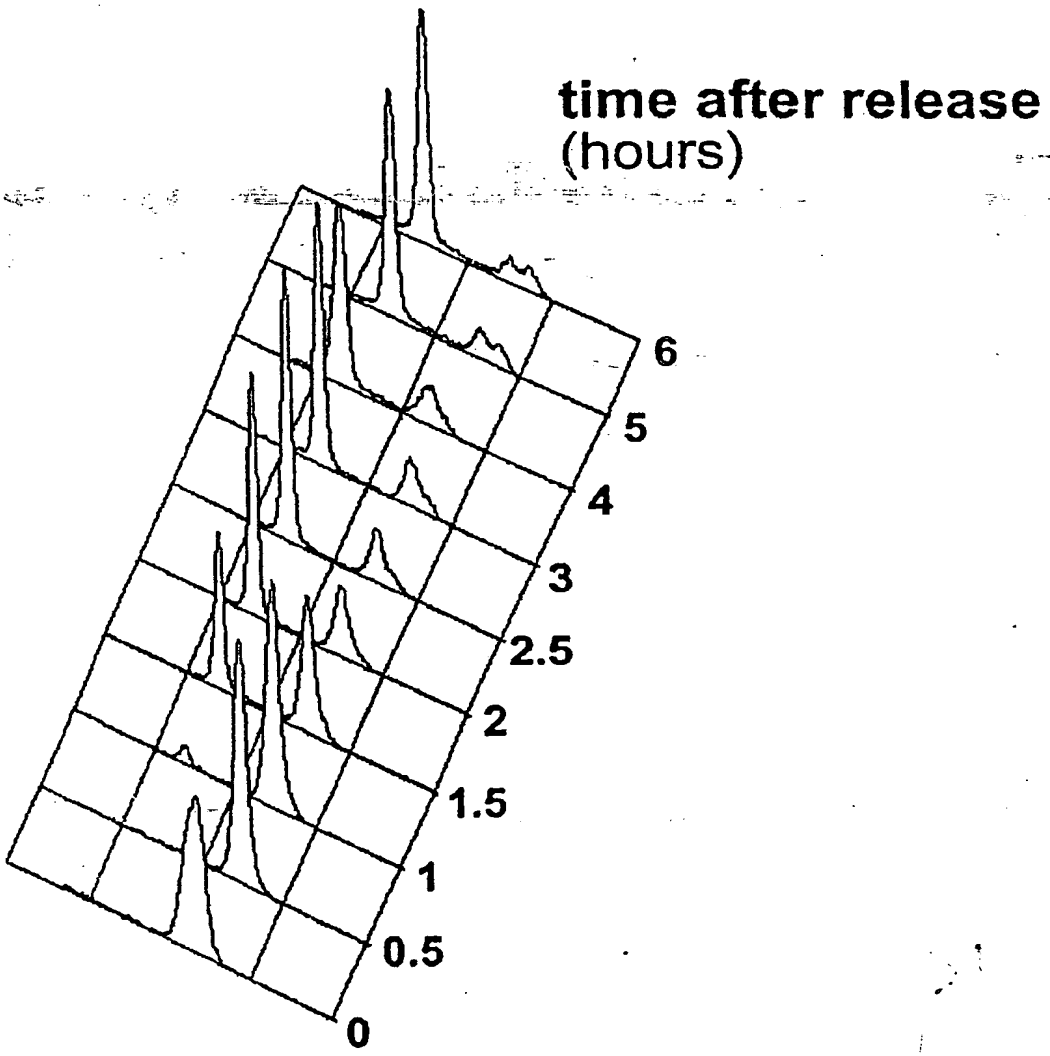
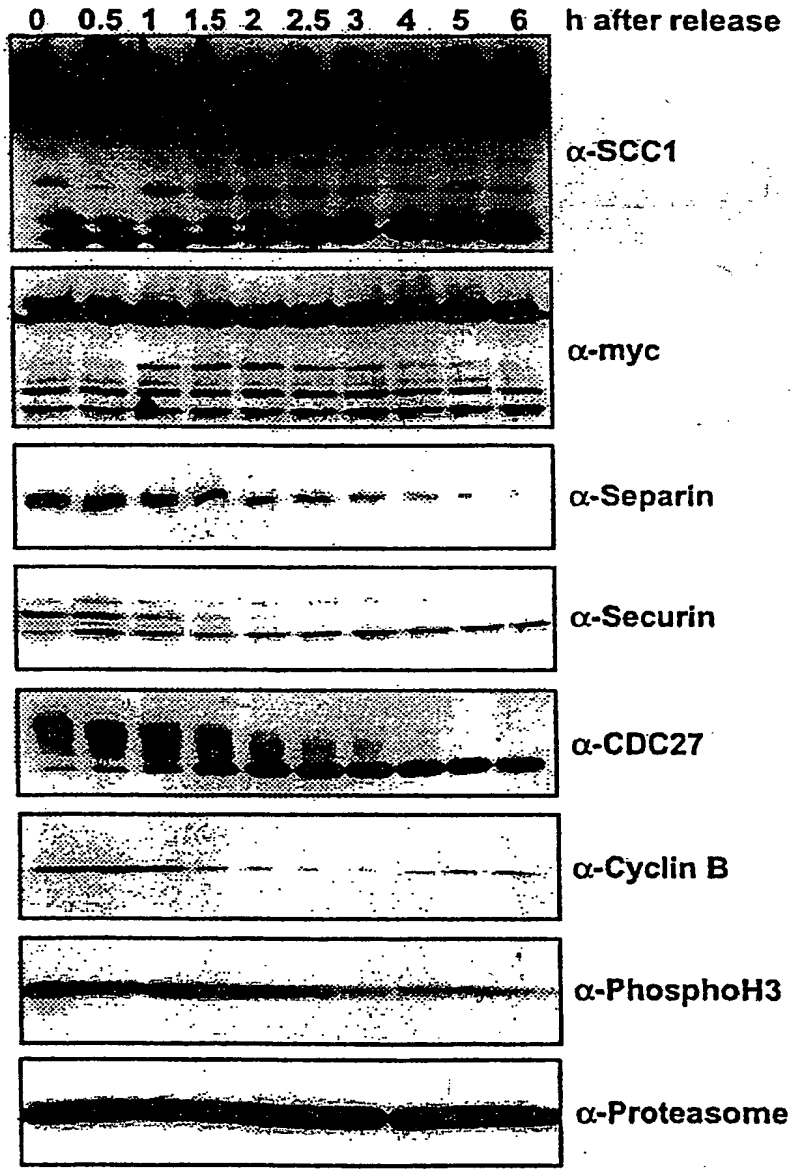


Fig. 12 b


0 0.5 1 1.5 2 2.5 3 4 5 6 h after release
α-SCC1
α-myc
α-Separin
α-Securin
α-CDC27
α-Cyclin B
α-PhosphoH3
α-Proteasome

23/24
Fig. 13

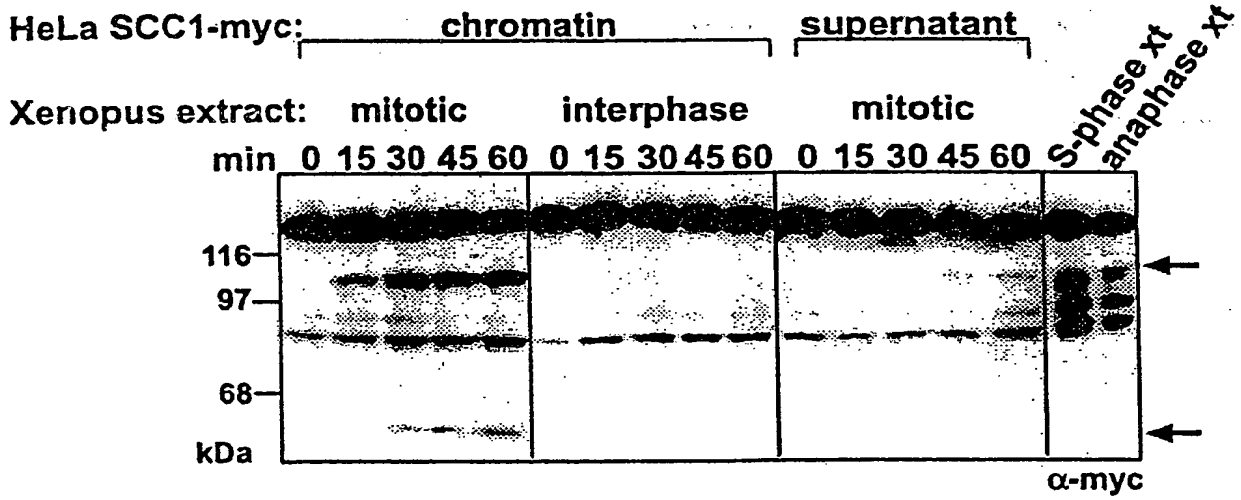
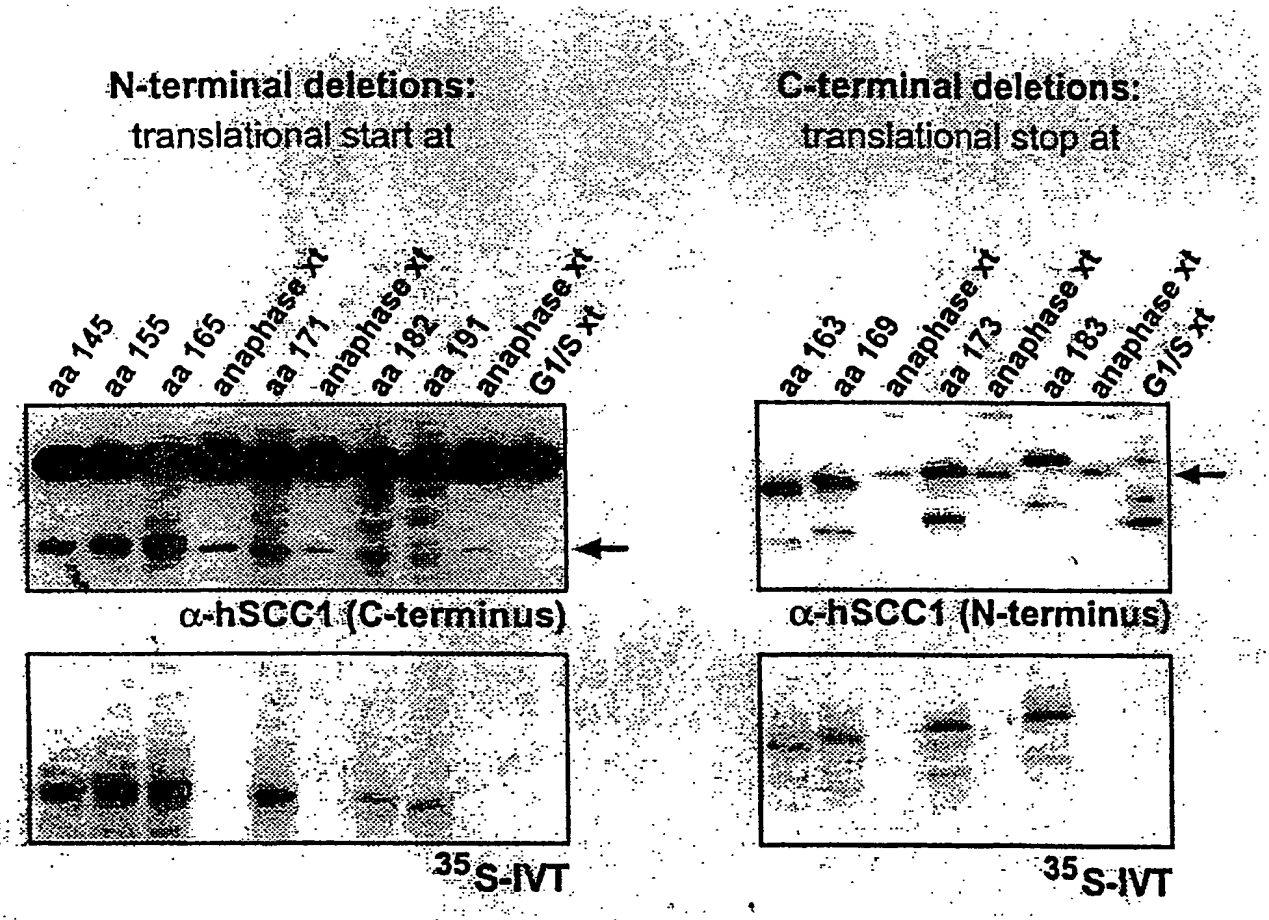

HeLa SCC1-myc:
chromatin
supernatant
Xenopus extract:
mitotic
interphase
mitotic
S-phase xt
anaphase xt
min 0 15 30 45 60 0 15 30 45 60 0 15 30 45 60
116
97
68
kDa
α-myc

Fig. 14


N-terminal deletions:
translational start at
C-terminal deletions:
translational stop at
aa 145
aa 155
aa 165
anaphase xt
aa 171
anaphase xt
aa 182
aa 191
anaphase xt
G1/S xt
aa 163
aa 169
anaphase xt
aa 173
anaphase xt
aa 183
anaphase xt
G1/S xt
α-hSCC1 (C-terminus)
α-hSCC1 (N-terminus)
35S-IVT
35S-IVT

SEQUENCE LISTING

<110> Boehringer Ingelheim International GmbH

<120> Compounds modulating sister chromatid separation and methods for identifying same

<130> 14047peptide

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<160> 1

<170> PatentIn Ver. 2.1

<210> 1
<211> 18
<212> PRT
<213> Homo sapiens

<400> 1

Met Asp Asp Arg Glu Ile Met Arg Glu Gly Ser Ala Phe Glu Asp Asp
1 5 10 15

Asp Met

INTERNATIONAL SEARCH REPORT

Inter ional Application No
PCT/EP 00/01183

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/55 A01H5/00 C12Q1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A01H G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 15749 A (PROTOTEK INC) 15 June 1995 (1995-06-15) page 1 -page 3, line 21 page 6, line 23 - line 26	10-12,15
X	WO 98 49190 A (CORTECH INC ;LEIMER AXEL H (US); SPRUCE LYLE W (US); CHERONIS JOHN) 5 November 1998 (1998-11-05) page 1 -page 2 page 25, line 19 - line 27	10-12,15

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 June 2000	29/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Fernandez y Branas,F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 00/01183

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CIOSK R ET AL: "An ESP1 /PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast." CELL, (1998 JUN 12) 93 (6) 1067-76., XP002118931 cited in the application the whole document	1-9
A	WO 98 27994 A (UNIV TEXAS) 2 July 1998 (1998-07-02) the whole document	1-3
A	CLARK D A ET AL: "PROTEASE INHIBITORS SUPPRESS IN VITRO GROWTH OF HUMAN SMALL CELL LUNG CANCER" PEPTIDES, vol. 14, no. 5, 1993, pages 1021-1028, XP002914836 the whole document	1,2
A	DATABASE EMBL 'Online! AC D79987, 1995 NOMURA N ET AL: "Prediction of the coding sequences of unidentified human genes. V. The coding sequences of 40 new genes (KIAA 0161-KIAA0200) deduced by analysis of the cDNA clones from human cell line KG1" XP002139909 abstract & DNA RES. Vol 3, 17-24, 1996	1-16
T	UHLMANN F. ET AL: "Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1" NATURE, vol. 400, July 1999 (1999-07), pages 37-42, XP002118932 the whole document	1-3

4

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 10-16 (partially)

Present claims 10-16 relate to a compound defined by reference to a desirable characteristic or property, namely the inhibition of "proteases with separin-like activity".

The claims cover all compounds having this characteristic or property, whereas the application provides no support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for the general inventive idea of the inhibition the so called "separin-like protease" for the uses defined in claims 10-15.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter ional Application No
PCT/EP 00/01183

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9515749 A	15-06-1995	US 5486623 A	23-01-1996
		AU 1266495 A	27-06-1995
		CA 2177495 A	15-06-1995
		EP 0731696 A	18-09-1996
		JP 9506368 T	24-06-1997
		US 5714484 A	03-02-1998
		US 5663380 A	02-09-1997
		US 5925772 A	20-07-1999
WO 9849190 A	05-11-1998	US 6004933 A	21-12-1999
		AU 7155698 A	24-11-1998
		EP 0979242 A	16-02-2000
		AU 3965199 A	08-11-1999
		WO 9954317 A	28-10-1999
WO 9827994 A	02-07-1998	AU 5384898 A	17-07-1998
		EP 0956032 A	17-11-1999

MOLECULAR AND CELLULAR BIOLOGY, May 1999, p. 3515–3528
0270-7306/99/$04.00+0

Vol. 19, No. 5

Rec8p, a Meiotic Recombination and Sister Chromatid Cohesion Phosphoprotein of the Rad21p Family Conserved from Fission Yeast to Humans

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Our work and that of others defined mitosis-specific (Rad21 subfamily) and meiosis-specific (Rec8 subfamily) proteins involved in sister chromatid cohesion in several eukaryotes, including humans. Mutation of the fission yeast *Schizosaccharomyces pombe rec8* gene was previously shown to confer a number of meiotic phenotypes, including strong reduction of recombination frequencies in the central region of chromosome III, absence of linear element polymerization, reduced pairing of homologous chromosomes, reduced sister chromatid cohesion, aberrant chromosome segregation, defects in spore formation, and reduced spore viability. Here we extend the description of recombination reduction to the central regions of chromosomes I and II. We show at the protein level that expression of *rec8* is meiosis specific and that Rec8p localizes to approximately 100 foci per prophase nucleus. Rec8p was present in an unphosphorylated form early in meiotic prophase but was phosphorylated prior to meiosis I, as demonstrated by analysis of the *mei4* mutant blocked before meiosis I. Evidence for the persistence of Rec8p beyond meiosis I was obtained by analysis of the mutant *mes1* blocked before meiosis II. A human gene, which we designate *hrec8*, showed significant primary sequence similarity to *rec8* and was mapped to chromosome 14. High mRNA expression of mouse and human *rec8* genes was found only in germ line cells, specifically in testes and, interestingly, in spermatids. *hrec8* was also expressed at a low level in the thymus. Sequence similarity and testis-specific expression indicate evolutionarily conserved functions of Rec8p in meiosis. Possible roles of Rec8p in the integration of different meiotic events are discussed.

Meiosis is an essential step in the sexual reproduction of eukaryotes. It serves to reduce the chromosome number from diploidy in the germ line to haploidy in the gametes. This is accomplished by two rounds of chromosome segregation, meiosis I and II, after a single round of DNA replication. During meiotic prophase, the replicated homologous chromosomes pair, recombination occurs between nonsister chromatids, and the resulting crossovers lead to chiasma formation in all bivalents. Chiasmata and sister chromatid cohesion are required for correct segregation of homologous chromosomes at meiosis I (reductional division). Throughout meiotic prophase, numerous events occur at the chromosomes in highly ordered fashion (for reviews, see references 37 and 68).

The events occurring at the DNA level are coordinated with many other cellular processes, and all are integrated into the meiotic cell cycle. Much additional research is required for the elucidation of the regulatory mechanisms governing meiosis. However, it is known that checkpoint proteins involved in the regulation of the mitotic cell cycle are also needed for checkpoint controls in meiosis (47). A number of protein kinases have important functions in meiosis. An example is the kinase encoded by the *Drosophila melanogaster mei-41* gene, which is homologous to the human *ATM* gene (30). Mutation of this gene affects the number and morphology of recombination nodules (12). The *CDC28*, *MEK1/MRE4*, *ESR2*, and *IME2* genes, coding for identified or putative protein kinases, have meiosis-specific functions in *Saccharomyces cerevisiae*: (19, 34, 44, 67, 74). A protein phosphatase was shown to interact with Red1, which is a component of the lateral elements of the *S. cerevisiae* synaptonemal complex (77, 83). A component of the lateral elements of the rat synaptonemal complex, SCP3, is multiply phosphorylated (43). SCP3 is identical to the Cor1 protein, which relocates from lateral elements to centromeres after anaphase I (20).

An important structural feature of chromosomes during mitotic and meiotic prophase is sister chromatid cohesion. It contributes to meiotic pairing and recombination of the homologous chromosomes. After crossover formation and degradation of the synaptonemal complex, the homologs are kept in alignment by the microscopically visible chiasmata, which do not resolve until anaphase I. If sister chromatid cohesion is resolved prematurely, chromosomes do not segregate properly, resulting in daughter cells with unbalanced genomes (for a review, see reference 52).

In several organisms, genes involved in meiotic sister chromatid cohesion have been identified. The *spo76* mutant of *Sordaria* shows precocious separation of sister chromatids and reduced meiotic recombination levels (55). Mutation of the *ord* gene of *D. melanogaster* also leads to premature separation of sister chromatids (7, 51). In *mei-S332* mutants of *D. melanogaster*, meiotic recombination and segregation of the homologs at meiosis I is normal. However, sister chromatids separate

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TABLE 1. Description of *S. pombe* strains used in this study

Strain	Genotype	Source or reference
PA3	*h⁻ ade6-52 pro2-1 leu1-32 ura4-D18*	This study
PA4	*h⁺ ade6-M26 arg3-124 leu1-32 ura4-D18*	This study
PA21	*h⁻ ade6-52 pro2-1 leu1-32 rec8::ura4 ura4-D18*	This study
PA22	*h⁺ ade6-M26 arg3-124 leu1-32 rec8::ura4 ura4-D18*	This study
PA32	*h⁻ leu1-32 rec8::ura4 ura4-D18*	This study
PA39	*h⁻ ade6-M210* / *h⁺ ade6-M216*	Strain collection, Bern
PA40	*h⁻ ade6-M210 leu1-32 rec8::ura4 ura4-D18* / *h⁺ ade6-M216 leu1-32 rec8::ura4 ura4-D18*	This study
PA41	*h⁻ ade6-M210 ura4-aim mei4-B2 ura4-D18* / *h⁺ ade6-M216 ura4-aim mei4-B2 ura4-D18*	This study
PA42	*h⁻ ade6-M210 mes1* / *h⁺ ade6-M216 mes1*	This study
PA43	*h⁹⁰ leu1-32 rec8::ura4 ura4-D18*	This study

precociously during anaphase I, leading to random segregation at meiosis II (35). Mei-S332 protein associates with centromeres in late meiotic prophase and disappears at anaphase II, when sister chromatids separate completely (36). A function similar to Mei-S332 was attributed to mouse Cor1 on the basis of its specific localization to centromeric regions from anaphase I to anaphase II (20, 53).

Loss of mitotic sister chromatid cohesion in *S. cerevisiae* requires degradation of Pds1p at the metaphase-to-anaphase transition (14). No homologs of Pds1p were identified in other organisms, but Cut2p of the fission yeast *S. pombe* may have a similar function (24). Recently, additional proteins involved in mitotic sister chromatid cohesion in *S. cerevisiae* were described (49). One of them, Scc1p, is related to Rad21p of the fission yeast *S. pombe* (8, 9). Scc1p binds to chromosomes during S phase, dissociates at the metaphase-to-anaphase transition, and is needed for sister chromatid cohesion near centromeres and in the chromosome arms. Binding of Scc1p to chromosomes is Smc1p dependent. The same protein, but named Mcd1p, was also identified in a screen for structural proteins of chromosomes and for proteins interacting with Smc1p, suggesting an additional role for Scc1p/Mcd1p in chromosome condensation (28). In addition to Rad21p, homologs of Scc1p/Mcd1p appear to exist in all eukaryotes, including *Homo sapiens* (reference 48 and this study).

The *rec8* gene of *S. pombe* was identified by screening for mutants with reduced meiotic recombination in the *ade6* gene (63). Point mutations in *rec8* reduce meiotic but not mitotic recombination and result in mutants with normal resistance to the DNA-damaging agents UV radiation and methyl methanesulfonate (17). Strong reduction of recombination was reported to be specific for a 2-Mb region around the centromere of chromosome III (18; but see below). Expression of *rec8* RNA was concluded to be meiosis specific based on meiosis induction in haploid *pat1* mutant cells (46). In mutants with a *rec8-110* point mutation, linear-element formation is defective and only amorphous aggregates of linear-element proteins were observed (54). The linear elements appearing in *S. pombe* prophase resemble axial cores (precursors of the lateral elements in the synaptonemal complex) of other eukaryotes (2). Bouquet formation was normal in *rec8-110* mutants, but pairing of chromosomes, studied by fluorescent in situ hybridization (FISH), was reduced. The strongly reduced spore viability was assumed to be a consequence of precocious separation of sister chromatids, which was observed in genetic assays and by FISH. It was concluded that linear-element formation contributes both to sister chromatid cohesion and to high meiotic recombination frequency (54).

Here we expand on the characterization of fission yeast Rec8p by performing an analysis of a new *rec8* allele, a gene deletion/disruption likely to create a null phenotype, and using an anti-Rec8p antibody in the study of Rec8p localization and Rec8p phosphorylation. We report the cloning and characterization of a novel human gene, *hrec8*, and present the results of experiments on complementation of the fission yeast *rec8* disruption strain by the human homolog. We describe in detail the *rad21/rec8* gene family. Finally, the results are discussed with respect to possible roles of Rec8p in the regulation of meiotic events.

MATERIALS AND METHODS

Strains, plasmids, media, and general methods. The genotypes of *S. pombe* strains used in this study are listed in Table 1. All *rec8::ura4* mutant strains were constructed from PA32 by genetic crosses. Plasmid pYL3 (46) contains a 3.9-kb *rec8* fragment subcloned in pSP2 (16). Plasmid pREP41-hrec8 was prepared by insertion of the full-length human *rec8* (*hrec8*) cDNA into the *NdeI* site of plasmid pREP41 (5).

General genetic methods and the standard media yeast extract agar (YEA) and malt extract agar (MEA) were as described previously (29). Minimal medium (MMA) consists of 0.67% Difco Nitrogen Base without amino acids, 1% glucose, and 1.8% agar; synthetic growth medium (GMA) consists of 0.17% Difco Nitrogen Base without amino acids, 0.375% sodium glutamate, 1% glucose, and 2% agar. EMM (a modified Edinburgh minimal medium), used for the complementation analysis of *rec8::ura4*, was prepared with 20 g of agar per liter (71). All growth factors were added to a final concentration of 100 mg/liter. For meiotic time course experiments, the synthetic minimal medium PM (*S. pombe* minimal) (6) and PM − N (PM without NH_4Cl) (86) were used.

***rec8* gene disruption.** To construct a *rec8* gene disruption mutant, a 3.9-kb *SacI* fragment from pYL3 (46) containing the *rec8* gene and flanking sequences was subcloned into pBluescript KS (Stratagene). From the resulting plasmid, pBSrec8S-1, a 1.5-kb *NsiI-NheI* fragment was replaced by a 1.8-kb *PstI-XbaI* fragment from pB4-3 containing the *ura4* marker gene (25) to yield the gene disruption plasmid pBSrec8S-1::ura4. From this plasmid, a 4.2-kb *SacI* fragment was used to transform the strain *h⁻ leu1-32 ura4-D18* by the lithium acetate method (33). Proper integration of the fragment into the genome was verified by Southern blot analysis.

Anti-Rec8p antibody. A 1.2-kb *rec8* DNA fragment covering the originally published open reading frame (ORF) from positions 742 to 1923 (46) was amplified by PCR to introduce a *BglII* and an *EcoRI* restriction site at the 5′ and 3′ ends of the fragment, respectively. This PCR product was ligated in frame into the *BamHI* and *EcoRI* sites of pGEX-2T (Pharmacia) to obtain a *rec8*-glutathione *S*-transferase (*rec8*-GST) fusion. The construct was verified by DNA sequence analysis.

Fusion protein was produced as described previously (78). Briefly, *Escherichia coli* DH5α cells were transformed with pGEX-2T-rec8 and selected for the presence of plasmid with ampicillin (50 μg/ml). Synthesis of fusion protein was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.1 mM. After further incubation for 3 to 4 h at 37°C, the cells were harvested and stored at −70°C. For preparation of *E. coli* extracts, approximately

4 g of cells was resuspended in 40 ml of phosphate-buffered saline (PBS) containing 2 mM EDTA, 100 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. The GST fusion protein was isolated from the cleared lysate by the addition of 2 ml of 50% slurry of glutathione-Sepharose 4B (Pharmacia). The fusion protein was eluted in 50 mM Tris-HCl (pH 8.0)–10 mM reduced glutathione, and eluted fractions were concentrated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The 72-kDa Rec8-GST fusion protein was isolated from the polyacrylamide gel and used for immunization.

Polyclonal antiserum was obtained by injecting a rabbit and three rats three times with approximately 200 μg of Rec8 fusion protein at 2-week intervals. Antisera were affinity purified by three rounds of adsorption to nitrocellulose Western blot strips (65) containing three different proteins (GST, *E. coli* heat shock protein GroEL, and Rec8 fusion protein). GroEL was a contamination in the preparation of Rec8 fusion protein as identified by protein sequencing.

Meiotic time courses and Rec8p immunofluorescence on nuclear spreads. The induction of meiosis, the preparation of nuclear spreads, and 4′,6-diamino-2-phenylindole (DAPI) staining of DNA were as described previously (2). Briefly, *S. pombe* PA39 diploid cells were induced to undergo meiosis by being shifted to nitrogen-free medium. Aliquots collected immediately after the shift (0 h) and at 2-h intervals thereafter were used to prepare spreads of nuclei. For flow-cytometric analysis, 1-ml aliquots of cells were fixed in 70% ethanol and stained with propidium iodide (Sigma) as described elsewhere (6). For immunofluorescence experiments, the wild-type diploid strain PA39 and the control diploid strain PA40 homozygous for the *rec8::ura4* deletion were used (Table 1). Slides with nuclear spreads stored at −70°C were soaked in PBS containing 0.1% Photo-Flo (Kodak) to remove the sucrose layer and then washed for 15 min in PBS containing 0.05% Triton X-100 and for 15 min in twofold-diluted blocking buffer (100 mM lysine, 3% nonfat dry milk, 0.05% Triton X-100, and 0.02% NaN_3 in PBS [pH 7.3]). The nuclear spreads were then blocked overnight in blocking buffer and subsequently probed with the first antibody (1:10 dilution of rabbit anti-Rec8p in blocking buffer) for 24 h. The slides were sequentially washed in PBS containing 0.1% Photo-Flo and PBS containing 0.05% Triton X-100 for 15 min each prior to incubation with the second antibody for 24 h (1:80 dilution of goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate conjugate in PBS [Sigma]). Before being mounted, the slides were washed once more for 15 min in PBS containing 0.1% Photo-Flo and for 15 min in PBS containing 0.05% Triton X-100, with two additional washes in H_2O for 5 min each. The chromatin was counterstained with DAPI in a Vectashield antifade solution (Vecta Laboratories Inc.). The slides were analyzed with an epifluorescence (Zeiss Axiovert) microscope. Foci were quantified with the UTHSCSA Image Tool software (version 1.27).

RNA extraction, Northern blot hybridization, and reverse transcription-PCR. Total RNA from *S. pombe* cells was prepared as described previously (26). Hybridization with a ^{32}P-labelled *rec8* or *byr1* (58) DNA probe was performed as specified by the manufacturer (Bio-Rad) in its standard hybridization protocol. The *rec8* probe was the same 1.2-kb DNA fragment used to construct pGEX-2T-rec8. The *byr1* probe (a gift from A. M. Schweingruber) was the 0.4-kb PCR fragment from positions 976 to 1387 in the ORF.

A 300-μg portion of total RNA from a meiotic time course (8 h after the shift) was used to prepare meiotic mRNA with the Oligotex poly(A)$^+$ mRNA isolation kit (Qiagen). First-strand *rec8* cDNA was synthesized from 1 μg of meiotic mRNA with an oligo(dT)$_{18}$ primer and Superscript reverse transcriptase (GIBCO). Reverse transcription-PCR was performed with the primers 5′-GGA AAAGGGAGGAATGGGAGTAATTTGG-3′ (positions 186 to 213 of *rec8*) and 5′-GTGAAAAGTTTCAAATGGCATCGGTGC-3′ (positions 2013 to 2039 of *rec8*). The resulting PCR fragment was subcloned into pGEM-T (Promega) and subjected to DNA sequencing.

Northern blot analysis of the human *rec8* mRNA was performed as described previously (48). A multiple human tissue blot was obtained from Clontech (no. 7754-1); hybridization and wash conditions were as specified by the manufacturer. The blots were hybridized with ^{32}P-labelled cDNA probes.

Immunoblotting, immunoprecipitation, and phosphatase treatment. For the preparation of *S. pombe* extracts, approximately 1 g of cells from different time points of a meiotic time course was suspended in disruption buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 13 mM β-mercaptoethanol), mixed with an equal volume of glass beads, and disrupted in a mini bead-beater. Following SDS-PAGE, the proteins were transferred to nitrocellulose and the filter was blocked overnight in TBST (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 0.05% Tween 20, 0.01% NaN_3) containing 5% nonfat dry milk. The filter was then incubated with the primary rat anti-Rec8 antibody (1:20 in TBST containing 5% nonfat dry milk) for 2 h. After three washes in TBST, the second antibody (1:500 dilution of rabbit anti-rat IgG peroxidase conjugate [DAKO] in TBST) was added for 2 h, and the mixture was washed three times in TBST and developed with the ECL chemiluminescent detection kit (Amersham).

For immunoprecipitation, Dynabeads M-280 (Dynal) were coated with affinity-purified rabbit anti-Rec8p antibodies and used for magnetic Rec8p purification from freshly prepared crude extracts of wild-type or *mei4* diploid cells, respectively, as described by the manufacturer.

Lambda protein phosphatase (New England BioLabs) was used in dephosphorylation experiments. Crude extracts from 1 g of meiotic wild-type cells (8 h after induction of meiosis) were prepared and used to immunoprecipitate Rec8p as described above. Beads containing Rec8p were washed twice in reaction buffer (50 mM Tris-HCl, 0.1 mM disodium EDTA, 5 mM dithiothreitol, 0.01% Brij 35 [pH 7.5]) and split into three portions. The first was used as control for immunoprecipitation and used directly for SDS-PAGE, the second was incubated in reaction buffer with 2 mM $MnCl_2$ for 1 h at 30°C (no phosphatase), and the third was incubated with 800 U of lambda protein phosphatase for 1 h at 30°C.

Cloning and sequence analysis of *hrec8*. General molecular biology procedures were essentially as described previously (69). Two 20-mer primers were designed based on the nucleotide sequence of EST T33286, whose translated product was homologous to the C-terminal end of *hHR21*^{sp} (48) (see Results). The primers had their 5′ ends at 102 nucleotides before and 140 nucleotides after the terminal nucleotide of the *hrec8* ORF and were used to amplify a 241-bp fragment of the *hrec8* gene from a human T-cell leukemia cell line cDNA (cDNA kindly supplied by Karin van Gool). This PCR fragment was used as a probe to isolate *hrec8* cDNA clones from a normalized, gridded human infant-brain plasmid library (79), kindly supplied by P. Heutink. These *hrec8* clones were sequenced on both strands. Amino acid sequences were aligned with the ESEE program (version 1.09e; supplied by Eric Cabot). An unrooted phylogenic tree (see Fig. 5C) was constructed based on the protein sequence alignments of the *rec8/rad21* homologs shown in Fig. 5B; a tree based on alignment of all full-length *rec8/rad21* proteins with the DNA Man program (version 3.2; Lynnon Biosoft, Quebec, Canada) gave very similar results (not shown). Trees were drawn with the PHYLIP software package (22); pairwise distances were calculated with the Dayhoff PAM 001 matrix. PEST sequences (66) were identified by using PEST-find at IMB Jena.

Complementation of *rec8::ura4* by the human *hrec8* gene. *S. pombe* PA43 was transformed with either pREP41 or pREP41-hrec8 by the lithium acetate method (33). Transformants were selected on EMM plates lacking leucine. Transformants were plated onto MEA and allowed to sporulate for 72 h at 25°C. Cell-free spore suspensions were prepared by incubation of cell material with β-glucuronidase (Sigma) at 22°C for 16 h. The spore titer was determined by counting. The percentage of viable spores was determined by plating on YEA and counting of the forming colonies.

S. pombe PA21 and PA22 were transformed with either pREP41 or pREP41-hrec8 by the lithium acetate method (33). Transformants were selected following growth at 30°C for 5 days on EMM plates containing adenine, proline, and arginine but lacking leucine. Individual colonies were streaked on the same medium and grown for 24 h at 30°C. Crosses were conducted at 25°C for 3 days on EMM plates containing adenine, proline, and arginine. Cell-free spore suspensions were prepared by overnight treatment of aliquots of the crossing material with snail digestive juice. Measurement of spore viability and intragenic and intergenic recombination were done by standard genetic methods. The mean values of three experiments were determined.

Chromosomal localization. FISH experiments were performed with biotinylated *hrec8* cDNA probes, hybridized to metaphase spreads of normal human lymphocytes, as described previously (62, 84). Chromosomes were banded with DAPI and actinomycin D and counterstained with propidium iodide in antifade solution.

Nucleotide sequence accession numbers. The GenBank accession numbers for the fission yeast and human *rec8* DNA sequences are AJ223299 and AF006264, respectively.

RESULTS

The phenotypes of a *rec8* gene disruption and reevaluation of the region specific reduction of recombination. A renewed analysis of the *rec8* gene led to extension of the ORF at both ends (see the legend to Fig. 1). This result led to the discovery of homology to other genes in the data banks (see below). A gene disruption strain was constructed by replacement of a large part of the *rec8* ORF by the *ura4* marker gene (Fig. 1) for comparison of its phenotypes with those of the *rec8-110* point mutation studied previously (54). Spore viabilities measured by random spore analysis were 12 and 20% in the disruption and point mutants, respectively. This difference is statistically significant but of doubtful relevance. The frequencies of intragenic and intergenic recombination were clearly reduced in comparison to those of the wild type, but no significant differences between disruption and point mutation crosses were found. At *lys7* (*lys7-1* × *lys7-2*) and at *ade6* (*ade6-M26* × *ade6-52*), the differences were at most threefold. In the three intervals *pro2-arg3*, *leu2-lys7*, and *ade6-arg1*, the values were two- to threefold lower in the *rec8::ura4* than in the point mutation crosses (data not shown). The cytological analysis revealed an absence of linear elements and a shortened mei-

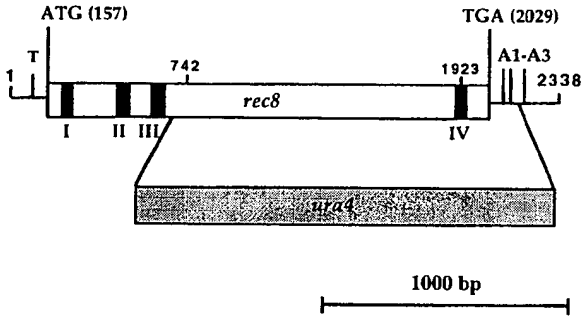


FIG. 1. Schematic diagram of a 2.3-kb fragment carrying the *rec8* gene and of the *rec8::ura4* insertion mutation. The open box indicates the ORF of the *rec8* gene. Solid boxes indicate the four introns at the following nucleotide positions: I at 213 to 255, II at 444 to 495, III at 593 to 644, and IV at 1895 to 1934. The putative TATA box (T), translation start and stop sites, and three potential polyadenylation signals (A1 to A3) are also indicated. In contrast to the originally published *rec8* sequence (742 to 1923) (46), the actual gene structure contains four introns that were first identified on the basis of the published splice consensus sequences for *S. pombe* (64) (reference 45 and data not shown). The existence of these introns was confirmed as described in Materials and Methods. The A of the ATG is position 157, and the A of the TGA is position 2029. To produce the *rec8::ura4* deletion mutant, the *ura4* marker gene was used to replace the fragment between the 5′ *Nsi*I and 3′ *Nhe*I restriction sites.

otic prophase in the *rec8-110* strain (54). Linear-element formation in *rec8::ura4* was impaired to a similar degree: no filaments and amorphous complexes of linear-element material were found. In contrast to the point mutation, the *rec8::ura4* strain showed no shortening of prophase in three independent time courses (data not shown). Shortening of the prophase in the point mutation strain may indicate a role of Rec8p in meiosis regulation. Alternative explanations, like shortening of prophase by an additional mutation, were not excluded.

It was previously reported that a study of the *rec8-110* point mutation showed that Rec8p is strongly required for activation of recombination in a 2-Mb region surrounding the *ade6* gene on chromosome III but only marginally involved at other regions of the genome (18). Here, we extend this recombination analysis both by using the *rec8* gene disruption mutant (*rec8::ura4*) and by testing new regions of chromosomes I and II. All data on intergenic recombination obtained with the point mutant (18) and our results from the gene disruption mutant are summarized in Fig. 2. The recombination frequencies obtained with the *rec8* gene disruption, the genotypes of the strains used, and the specific methods applied are available upon request.

We observed the strongest reduction of recombination (approximately 300-fold) in crosses with *rec8::ura4* at the *ade6–arg1* interval on chromosome III, confirming the published results (18). On chromosomes I and II, the strongest reductions of recombination were also observed in the vicinity of the centromeres. The centromere-spanning interval *tps13–leu1* and the centromere-proximal interval *ade7–his3* on the short arm of chromosome II showed reductions of 100- and 300-fold, respectively. These values are comparable to those obtained for chromosome III. On chromosome I, the longest chromosome, the centromeric interval *aro5–lys1* showed a 30-fold reduction. Moderate but significant reductions were found in the arms of chromosome I and close to the telomeres of chromosomes I and II.

Rec8p is localized in foci in the nucleus. When intact wild-type cells from vegetative or meiotic cultures were fixed and treated with affinity-purified anti-Rec8p antibody, no signal was detected unless Rec8p was overexpressed from a plasmid, in which case the signal was confined to the nucleus (data not shown).

Gentle lysis of cells and spreading of nuclei on surfaces were used previously for the analysis of linear-element formation and chromosome pairing (2, 54, 70). Thus, spread meiotic

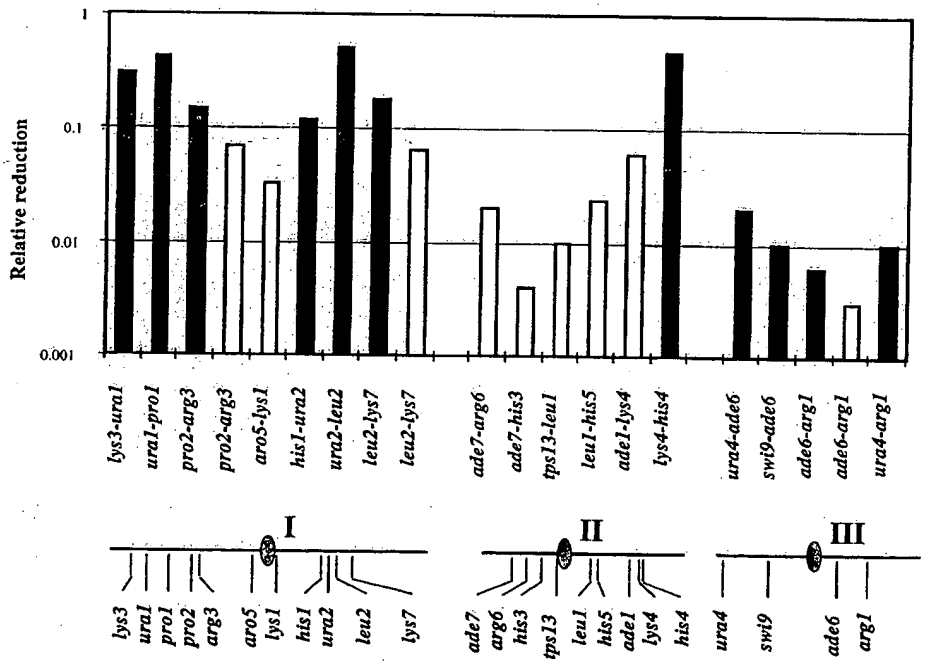


FIG. 2. Meiotic recombinant frequencies in *rec8* mutants. For each interval, the frequency is expressed relative to the one measured in the *rec*$^+$ cross. The solid bars represent the data from reference 18, and the open bars represent those obtained from *rec8::ura4* crosses. Recombination frequencies were measured twice in every interval in the *rec*$^+$ and the *rec8::ura4* backgrounds. The approximate positions of the genes on the chromosomes are shown below the histogram.

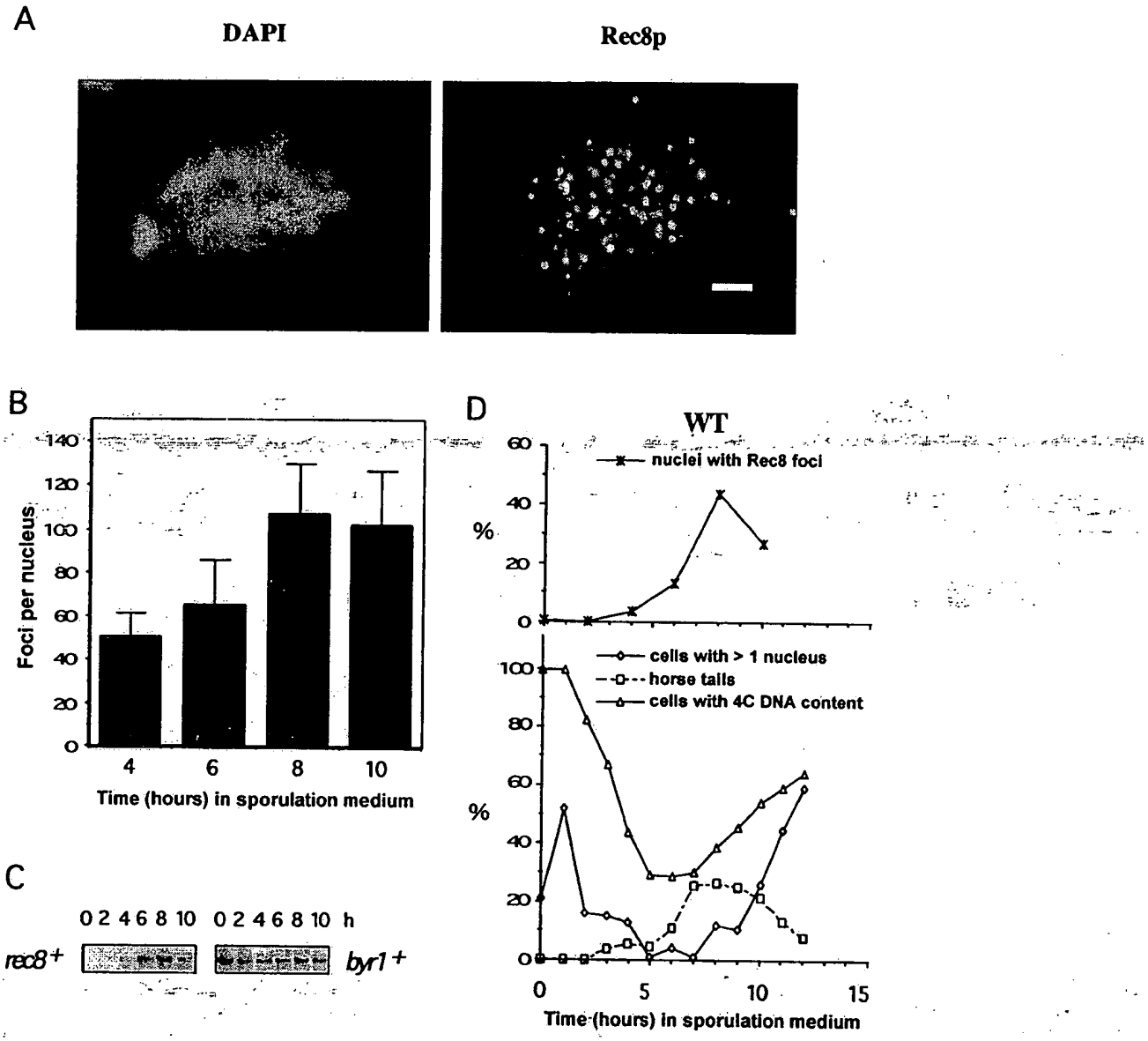


FIG. 3. Immunolocalization of Rec8p-staining foci and *rec8* mRNA expression in relation to a time course of cytological events during wild-type meiosis. Nuclear spreads were prepared from wild-type (PA39) diploid cells 0, 2, 4, 6, 8, and 10 h after transfer to meiosis medium, and all data are from the same meiosis time course experiment. (A) Example of a spread wild-type nucleus 8 h after induction of meiosis. Rec8p was visualized with an affinity purified polyclonal anti-Rec8p antibody (right), and the DNA was counterstained with DAPI (left). Bar, 2 μm. (B) Quantitation of Rec8p-staining foci during meiosis. The number of foci was determined in samples of 30 well-spread nuclei at the indicated time points. The error bars indicate 1 standard deviation. (C) Induction of *rec8* mRNA expression during wild-type meiosis. Northern blots of 20 μg of total RNA at the indicated time points were hybridized with a *rec8*-specific probe (left) and later hybridized to a *byr1*-specific cDNA probe to verify loading (right). (D) Timing of cytological events during wild-type (WT) meiosis. The number of horse-tail nuclei, which are markers of meiotic prophase, was determined by DAPI staining, and the percentage of cells with 4C DNA content was measured by flow cytometry. Cells with more than one nucleus represent the percentage of the cells that have completed the first meiotic division. The increase in the number of cells containing two nuclei 1 h after induction of meiosis is due to the mitotic division of cells in G_2 phase. This last mitotic division must occur before cells can enter meiosis from the G_1 phase (21).

nuclei from wild-type diploids were treated with affinity-purified anti-Rec8p antibody (see Materials and Methods). Fluorescence microscopy revealed that Rec8p is localized in foci throughout the spread nuclei. Meiotic time course experiments were then performed with the wild-type diploid strain PA39 and with the *rec8::ura4* disruption diploid strain PA40. An example of the analysis of meiotic landmarks, the presence of *rec8* mRNA, and the subnuclear localization of Rec8p (quantitation of Rec8p foci) are presented in Fig. 3.

Rec8p was localized to distinct foci of different intensities within the nucleus (Fig. 3A). The intensity of Rec8p foci was dependent on the focal plane. No staining above background was observed on spreads of a time course of the diploid PA40 (*rec8::ura4* [data not shown]). In general, the foci were round. The few elongated foci may represent two or more unresolved structures. Some adjacent foci (three to seven foci) resembled pearls on a string, while other foci were more widely separated and scattered in the same nucleus. We quantified the Rec8p foci per individual nucleus during a meiotic time course experiment (Fig. 3B). The average number of foci per nucleus in-

creased from about 50 at early prophase (4 h after the shift to meiotic conditions) to more than 100 at late prophase (8 and 10 h). Similar results were obtained in a second independent time course. Foci first appeared after 4 h, when premeiotic DNA replication had started and when cells with elongated and deformed horse-tail nuclei (typical of meiotic prophase) started to accumulate (Fig. 3D). At 8 h, the fraction of nuclei with foci reached its maximum (42%). After 10 h, reliable scoring of nuclei with foci and quantitation of foci within single nuclei were no longer possible due to poor spreading properties of the nuclei (spore formation).

The number of nuclei carrying Rec8p foci correlated with the relative amount of *rec8* mRNA at the different time points (Fig. 3C and D). Rec8p mRNA expression was previously shown to be specifically induced in haploid *pat1* meiosis (46). The size of the transcript (2.0 kb) is consistent with the *rec8* ORF (1,683 nucleotides) shown in Fig. 1.

The percentage of nuclei with foci was brought into the context of classical landmarks of fission yeast meiosis (Fig. 3D). The transition from mitotic G_2 to G_1 cells followed by premeiotic S phase was visualized by flow cytometry for DNA content. The large number of cells with two nuclei 1 h after induction of meiosis represents the final mitotic division before cells entered the meiotic prophase. The number of cells with more than one nucleus was smallest from 5 to 7 h and then increased again, indicating the onset of meiosis I. At 12 h after induction of meiosis, roughly 60% of the cells had completed the first meiotic division. The abundance of nuclei with foci coincided fully with the presence of nuclei with an extended shape (horse-tail nuclei), which appear to be due to the vigorous movement of prophase nuclei (13, 81).

Rec8p is phosphorylated during prophase and persists beyond meiosis I. To further examine Rec8p expression during meiosis, time course Western analysis and immunoprecipitation experiments were performed with both wild-type diploid PA39 cells and diploid cells homozygous for mutations that block meiosis at specific stages. The particular PA39 culture used for the experiment in Fig. 4A was somewhat delayed compared to the cultures used for the study of Rec8p foci (Fig. 3). Rec8p first appeared 6 h after induction of meiosis, when the first horse-tail nuclei also became visible (data not shown), and grew more abundant at the later time points. At 6 h, Rec8p was visible as a single band of 87 kDa. At 8 h, an additional band migrating at 95 kDa became apparent and its intensity increased (10 and 12 h). After 12 h, protein extraction became difficult due to the presence of high percentages of spores. At 12 h, 40% of cells had performed the first meiotic division and fewer than 10% of the cells were still in meiotic prophase (horse-tail nuclei [data not shown]). The apparently large amount of Rec8p in the extract from cells harvested at 12 h indicated to us that Rec8p may persist beyond meiosis I (see below).

The appearance of an additional protein band suggested that Rec8p is modified during meiotic prophase. To test whether the additional Rec8p band was due to phosphorylation, Rec8p was immunoprecipitated from extracts of wild-type cells and treated with phosphatase. Figure 4B shows that the low-mobility Rec8p band was removed by this treatment, suggesting that Rec8p is phosphorylated during meiotic prophase. The high-mobility form, appearing first as a single band, did not change upon phosphatase treatment (control experiment; results not shown).

The data in Fig. 4A indicate that Rec8p phosphorylation occurs prior to the first meiotic division. If this is the case, mutant cells blocked before meiosis I are expected to contain phosphorylated Rec8p. Thus, the protein was immunoprecipitated from extracts of *mei4* diploid cells, which are blocked in meiotic prophase at the stage with fully developed linear elements (2). In contrast to wild-type meiosis, no cells in the *mei4* culture had two nuclei (indicated by DAPI staining) 10 h after induction of meiosis, confirming that the *mei4* cells were blocked in prophase. Both versions of Rec8p were apparent as distinct bands; in addition, smaller proteins which may be degradation products were present (Fig. 4C). This result is consistent with Rec8p becoming phosphorylated prior to meiosis I.

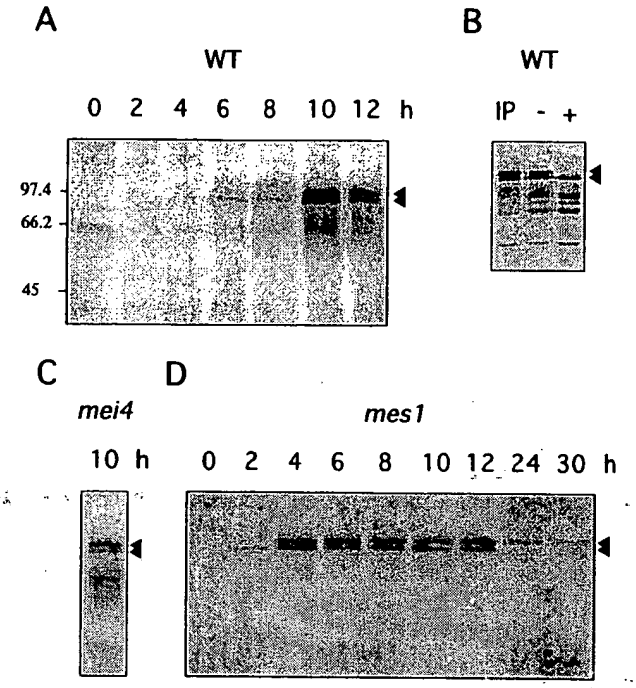


FIG. 4. Expression and phosphorylation of Rec8p during meiosis. (A) Protein extracts were prepared at different time points of a meiosis time course of the wild-type (PA39; WT) diploid. Aliquots of 300 μg were subjected to SDS-PAGE and Western blot analysis with affinity-purified anti-Rec8p antiserum. Arrowheads indicate the two forms of Rec8p. (B) Phosphatase treatment of Rec8p from wild-type (WT) cells (strain PA39) 8 h after induction of meiosis. IP, immunoprecipitate; −, no phosphatase treatment; +, phosphatase treatment. (C) Immunoprecipitation of Rec8p from *mei4* (PA41) cell extracts 10 h after a shift to sporulation medium. (D) Meiotic time course of the *mes1* diploid strain PA42. Protein extracts were prepared at the indicated time points, and aliquots of 150 μg were subjected to SDS-PAGE and Western blot analysis with affinity-purified rat anti-Rec8p antiserum.

Persistence of Rec8p beyond meiosis I is expected to lead to the presence of the protein in cells blocked after meiosis I and before meiosis II. Diploids homozygous for a *mes1* mutation are blocked after the first and before the second meiotic division (11, 74). The same Rec8p double-band pattern was observed in wild-type cells (Fig. 4A) and *mes1* cells (Fig. 4D) up to 12 h after the medium shift. In the *mes1* time course, horse-tail nuclei were already visible 2 h after the shift and disappeared completely after 12 h. At 24 h, all of the cells which had entered meiosis (32%) had performed the first meiotic division, as determined by DAPI staining. At 30 h, the percentage of cells with two nuclei was also 32%. This shows that in *mes1* mutants, by 24 h the cells were fully blocked after the first meiotic division. At these time points, the low-mobility Rec8p predominated in Western blots of crude extracts (Fig. 4D). Upon immunoprecipitation, both protein forms were detectable, but for unknown reasons the low-mobility form could not be abolished by phosphatase treatment (data not shown). The

observations made with the *mes1* mutant are consistent with persistence of Rec8p after meiosis I.

Cloning and sequence analysis of a human *rec8* gene. Since the conservation of the *rad21* class of genes extended from yeasts to mammals and *rec8* genes existed in both budding and fission yeast species (see below), it was likely that *rec8* was also conserved in mammals. A human expressed sequence tag, T33286, was identified by its homology to the human *rad21* homolog, *hHR21*^{sp} (for human homolog of *rad21*, *S. pombe* [48]) on BLAST (1) database searching. A cDNA fragment corresponding to the C-terminal region of this new human gene, which we denote *hrec8*, was amplified from a human T-cell leukemia cDNA pool and used to screen a cDNA library from which full-length *hrec8* cDNA clones were obtained.

hrec8 corresponded to an ORF of 1,641 nucleotides. Consistent with the generally low mRNA expression of this gene (see below), the sequence around the initiation codon did not conform strongly to the Kozak consensus (41); however, its translation start site was likely to be the correct one because of its highly conserved nature (below) and the presence of stop codons in all three reading frames in the 5′ untranslated region (5′ UTR) (data not shown). The 5′ UTR was at least 477 nucleotides long, while the 3′ UTR was 160 nucleotides (data not shown). The translation of *hrec8* is shown in Fig. 5A, aligned with the full-length fission yeast Rec8p, a putative Rec8p homolog in budding yeast (Rec8^{sc}; see below), and the founding member of the *rad21/rec8* gene family, Rad21p.

Rec8p and its homologs are clearly sequence related to Rad21p and its homologs (Fig. 5B). To distinguish between previously reported Rad21-related proteins and those described in this paper, the new proteins described here are referred to as the *rec8* members of the *rad21/rec8* gene family. Sequence homology is higher at the N- and C-terminal ends of the proteins, where all proteins of the family are around 30% identical. Overall, Hrec8p had 49% similarity and 26% identity to hHR21^{sp} (data not shown).

The PROSITE protein motif library failed to reveal major structural motifs, indicating a particular biological function for Hrec8p or related proteins. The unusual alternating basic-acidic region in the C-terminal region of the hHR21^{sp} protein (see Fig. 2A of reference 48) was absent from the *hrec8* product. Overall, Hrec8p is, like hHR21^{sp}, an acidic protein (pI 5.0), but there were dramatic variations in charge and pI across Hrec8p. Another notable feature of Hrec8p was its unusually high proline content of 14.3%. Like fission yeast Rec8p, which had conserved basic residues at amino acids 284 to 290, Hrec8p contained a stretch of 6 arginine residues preceded by a long stretch of prolines (amino acids 298 to 304; Fig. 5A). This basic region, conserved in *rad21/rec8* family homologs from other species (see Fig. 2 of reference 48), could represent a nuclear localization signal. Consistent with cell cycle regulation of these proteins by proteolysis (28, 49), potential PEST sequences (66) were identified in the central, less highly conserved regions of all the Rad21/Rec8 family proteins (data not shown).

Phylogenetic analysis of *rad21/rec8* gene family. A phylogenetic comparison of the Rec8 and Rad21 proteins from the different eukaryotes was performed. Overall, the estimated phylogenetic relationships between the Rec8/Rad21 proteins from the different species (indicated by the lengths of the lines in Fig. 5C) are consistent with those observed for other groups of orthologous and paralogous genes; i.e., they are congruent with the evolutionary relationship of the various species (32, 76, 82). There is no clear distinction between the *rad21* and *rec8* members of the gene family based on phylogeny. The common root of the two nematode proteins and their proximity to human *rad21* indicates that unlike in the other species examined to date, there are two *rad21* genes in *Caenorhabditis elegans*. However, no genes with sequence homology closer to *rec8* than to *rad21* have yet been identified in that species, although its sequence analysis has been completed (82).

Chromosomal localization and mRNA expression of *hrec8*. By using FISH, *hrec8* was mapped to chromosome 14q11.2-12 (Fig. 6A). This locus is not apparently implicated in human disease syndromes. The mRNA expression of *hrec8* was examined in different mammalian tissues. On blots prepared with total RNA, a 2.4-kb mouse *rec8* mRNA species, consistent with the size of the *hrec8* ORF, was detected only in testis cells (Fig. 6B, top left) and not in other tissues. Mouse testis tissues were fractionated into meiotic and postmeiotic compartments (spermatocytes and spermatids, respectively [27]). In contrast to the pattern seen for the related *mHR21*^{sp} gene (48), weak *mrec8* expression was detected in meiotic cells and greater expression was detected in postmeiotic spermatids (Fig. 6B, top middle). In addition, a multiple-human-tissue poly(A)$^+$ RNA blot was hybridized with an *hrec8* probe. Although not detected on the total-RNA blot, the *hrec8* mRNA level was increased in thymus tissue and, unlike in testis tissue, there was an additional 3.2-kb mRNA species (Fig. 6B, top right). Expression was not detectable in other tissues, including some with high cellular proliferation. This indicates that increased thymic and testicular *mrec8* expression was unlikely to simply reflect cellular proliferation in these tissues. We conclude that *hrec8* mRNA expression is increased in meiotic as well as postmeiotic testis cells and is also detectable in thymus cells.

Experiments on complementation of fission yeast *rec8::ura4* by human *hrec8* cDNA. Sequence similarity was demonstrated for fission yeast and human Rec8p, and both genes were expressed in meiosis. If the two proteins have common functions, hRec8p might be able to substitute for Rec8p function in *S. pombe*. Two sets of experiments were performed. The homothallic strain PA43 with the *rec8::ura4* disruption was transformed with pREP41 (control) and pREP41-hrec8. Individual transformants were brought to conjugation and sporulation, and the resulting spores were checked for viability. Untransformed PA43 showed a low spore viability of 9% compared to that of a *rec8* wild-type strain. The vector alone had no effect after transformation into PA43, but pREP41-hrec8 increased spore viability to 48% (normalized to that of the *rec8* wild-type strain [data not shown]).

Complementation of spore viability is expected to be due to restoration of high recombination frequency. Therefore, the heterothallic strains PA21 and PA22, suitable for recombination analysis, were transformed by pREP41 (control) and pREP41-hrec8. In this experiment, crossing the two untransformed strains resulted in low (18%) spore viability while transformation with the vector yielded 24% spore viability. Transformation with pREP41-hrec8 increased the spore viability to 38%. These results are qualitatively similar to those described above and may indicate partial complementation of the reduced spore viability phenotype by Hrec8p (data not shown). Neither intragenic recombination measured between the *ade6-M26* and *ade6-52* alleles nor intergenic recombination examined in the *pro2-arg3* interval was significantly different from that in crosses of the untransformed strains (data not shown). These results show that the partial complementation of spore viability may be of doubtful significance.

DISCUSSION

In this study, we have further elaborated the function of the fission yeast *rec8* gene, whose product is a phosphoprotein

A

FIG. 5.

critical for meiotic sister chromatid cohesion and correct chromosome segregation. We describe the sequence conservation of *rec8* in humans and present evidence that *hrec8* is the human homolog of fission yeast *rec8*. We also demonstrate that the *rec8* class of genes shows sequence homology to the *rad21* class of mitotic, cell cycle-regulated phosphoproteins involved in sister chromatid cohesion, chromosome condensation, and DNA double-strand break repair. The findings suggest that *rad21* and *rec8* are the mitotic and meiotic members, respectively, of a new gene family involved in multiple aspects of DNA metabolism.

Here we present a unifying hypothesis to explain the observations on Rec8p. In this context, some special features of fission yeast meiosis need to be kept in mind. *S. pombe* maintains the bouquet structure of chromosomes throughout meiotic prophase, with concomitant absence of a fully tripartite synaptonemal complex and of crossover interference (for reviews, see references 37, 39, and 68). The retention of linear

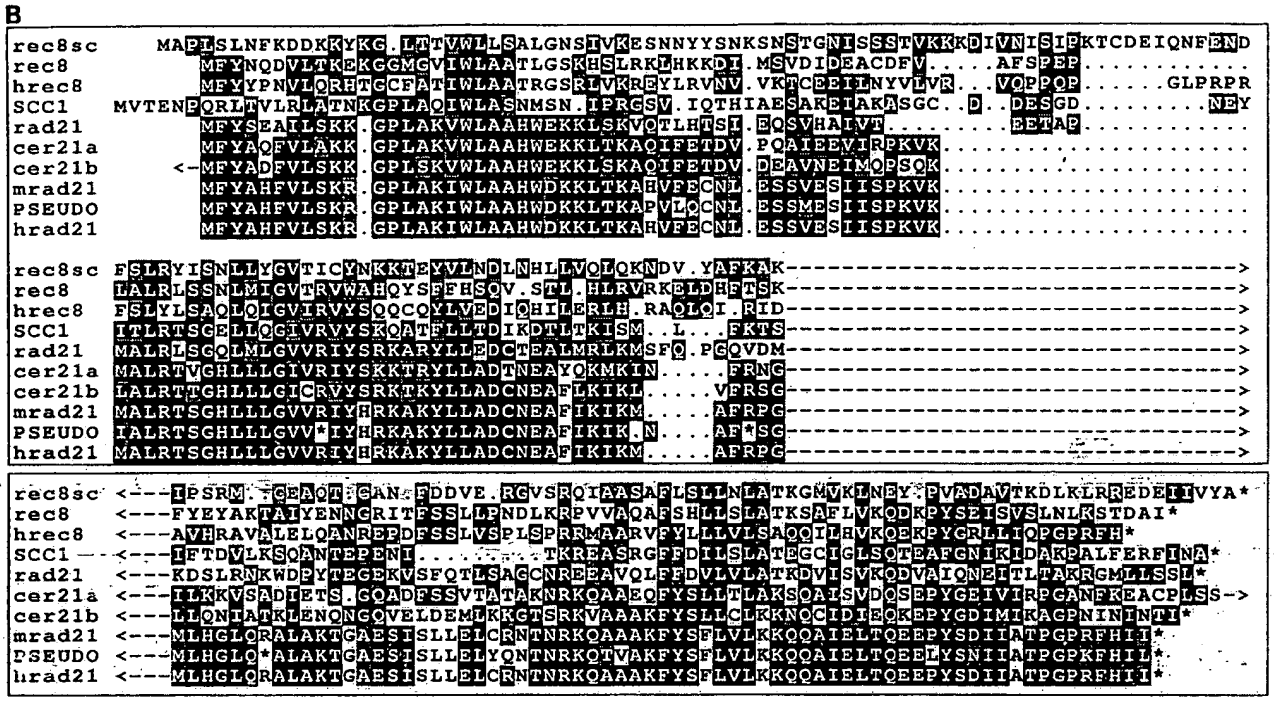
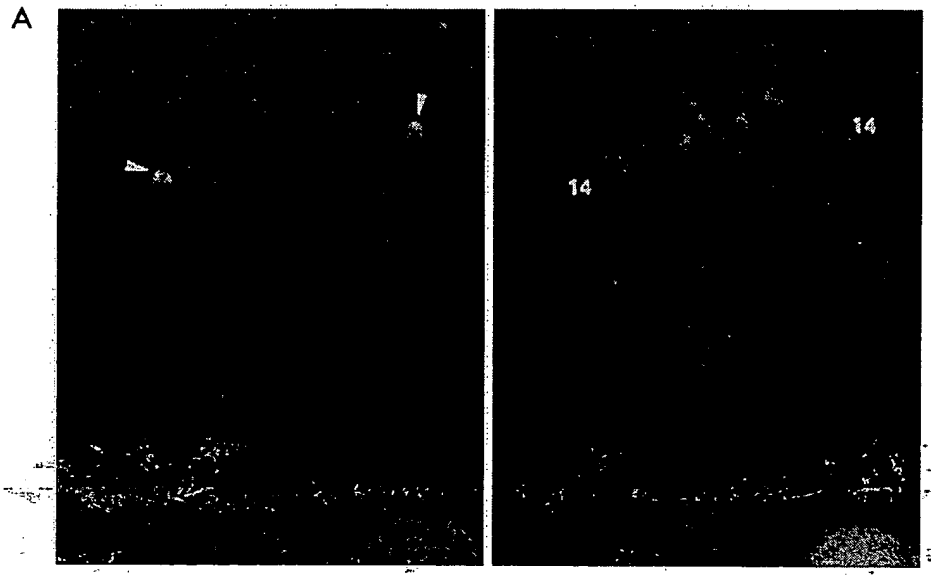


FIG. 5. *rad21/rec8* gene family. (A) Alignment of the predicted amino acid sequence of *S. pombe* Rec8p with human Rec8p, an *S. cerevisiae* Rec8p homolog (Rec8p^{sc}), and the founding family member, Rad21p. Letters in black boxes represent identical amino acids in at least two species, whereas those in gray boxes represent similar (P, A, G, S, and T; E, D, N, and Q; V, I, L, and M; F, W, and Y; R, K, and H) amino acids. Gaps introduced into the sequences for alignment optimization are shown as dots. Numbers denote amino acid numbers in the sequence. (B) Alignment of the conserved N- and C-terminal amino acid regions (top and bottom, respectively) of *rad21/rec8* gene family members from different species. Sequences shown here are as follows: rec8sc, *S. cerevisiae* Rec8p homolog (GenBank accession no. U31900); rec8 (reference 46 and this study); hrec8 (present study); SCC1, *S. cerevisiae* Rad21p homolog (28, 49) (GenBank U23759); rad21, *S. pombe* Rad21p (8); cer21a and cer21b, first and second *C. elegans* Rad21p homologs (reference 48 and this study) (GenBank U40029 and U38377, respectively); mrad21 and hrad21, mHR21^{sp} and hHR21^{sp} (mouse and human Rad21p homologs) (48) (GenBank X98293 and X98294, respectively); and PSEUDO, hHR21^{sp} pseudogene on chromosome X (GenBank HSU85A3). Rec8^{sc}, Rec8p, and hRec8p are the *S. cerevisiae*, *S. pombe*, and human Rec8 proteins that are the subject of this publication. Amino acid shading and general features of the alignment are as in panel A. Regions of less highly conserved sequence exist between the blocks and are indicated by dashes and arrowheads. The arrowhead at the end of cer21a represents 22 nonconserved amino acids at its C terminus. The arrowhead at the beginning of cer21b represents 55 nonconserved amino acids at its N terminus. (C) Unrooted phylogenetic tree of Rad21 and Rec8 proteins from different species. Evolutionary distances between the proteins from different species are indicated by the lengths of the lines in the figure. The sequences represented here are the same as in panel B, except that for reasons of clarity, the mHR21^{sp} protein (and the pseudogene) are not on the tree because of their evolutionary proximity to hHR21^{sp}p.

elements resembling the axial cores of synaptonemal complex indicates that these structures are of fundamental importance for meiotic recombination and chromosome segregation. During prophase of fission yeast meiosis the horse-tail nuclei move continuously from end to end of the cylindrical cells. At the leading end of the elongated nuclei, all telomeres are clustered to form and maintain the bouquet structure of chromosomes (13, 81). Disruption of the integrity of the spindle-pole body and/or telomere clustering by mutation leads to reduction of meiotic intra- and intergenic recombination frequencies (15, 59, 73). When nuclear movement was abolished by mutation of a motor protein required exclusively for horse-tail nucleus movement, recombination was also reduced (31). In all cases, about a fivefold reduction of recombination frequencies resulted.

The primary functions of Rec8p: a working hypothesis. We propose that freshly synthesized Rec8p binds to the sites of initiation of recombination that are a subset of the sites of early pairing between related DNA sequences (re)established immediately after premeiotic DNA replication. It is likely that at this early stage, ectopic interactions between repeated sequences on different chromosomes occur as well as truly homologous DNA contacts. Little is known about the mechanisms involved in the formation of the first contacts between homologous chromosomes and the resolution of ectopic interactions. In *S. cerevisiae*, early contacts between homologous chromosomes occur before double-strand breaks initiate recombination, and it was proposed that assembly of the recombination initiation complex occurs in a succession of events at sites of sister chromatid cohesion (for reviews, see references 37 and 68).



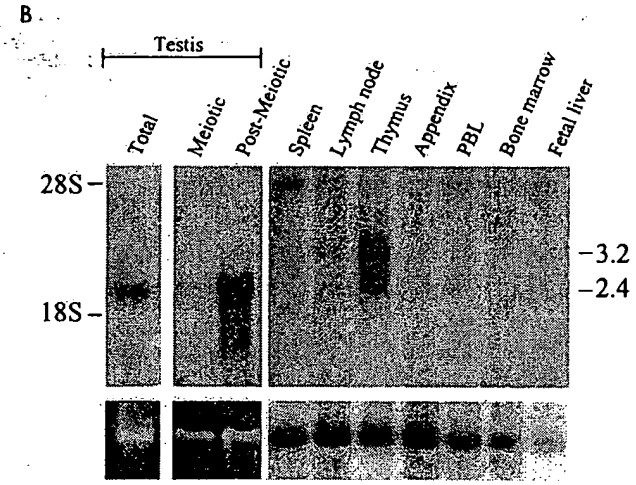


FIG. 6. Chromosomal assignment and tissue specificity of mammalian *rec8* mRNA expression. (A) FISH was used for *hrec8* chromosomal assignment; biotinylated full-length *hrec8* cDNA was hybridized with human metaphase spreads. (Left) Specific double-hybridization signals on chromosome 14q11.2-12 (arrowheads). (Right) DAPI staining of the same metaphase spread. (B) To examine mammalian *rec8* mRNA expression, Northern blots of RNA from various mouse and human tissues and different testis fractions were hybridized with an *hrec8* cDNA probe (top). (Top left) Single lane from a previously reported total RNA blot of mouse testis (Fig. 4 of reference 48); since no transcripts were detected in other tissues (thymus, brain, muscle, kidney, heart, liver, spleen, and ovary), these lanes are not shown here. (Top middle) Murine testis fractions representing meiotic (spermatocytes) and postmeiotic (a mixture of round and elongating spermatids) fractions. (Top right) Clontech multiple-human-tissue blot (no. 7754-1). There was some cross-hybridization of the *hrec8* probe with trace amounts of 28S rRNA, as previously observed by us (data not shown). (Bottom left and middle) Ethidium bromide staining of rRNA indicating lane loading. (Bottom right) The multiple tissue blot was rehybridized with a β-actin control for lane loading. The positions of the 28S and 18S rRNA bands are shown on the left, while the transcript sizes (in kilobases) are indicated on the right.

Rec8p binding to the developing recombination initiation complex is proposed to be required for the start of linear-element polymerization. Linear elements, in turn, are proposed to contribute to extension and stabilization of homologous chromosome pairing and to maintenance of recombination intermediates once initiation has occurred. In particular, we suggest that linear-element formation is important in chromosome regions far from the telomeres. Rec8p binding to early homologous contacts is also proposed to enhance sister chromatid cohesion directly, or indirectly by promotion of linear-element polymerization.

The phosphorylation of Rec8p may play a structural role in organizing the chromosomes during prophase (e.g., repulsion of DNA backbones). Alternatively, or in addition, phosphate groups may be introduced in response to completion of specific steps of the pairing and recombination pathway, in order to signal their occurrence to other components of the nucleus. Another regulatory function of the kinase(s) phosphorylating Rec8p may be the promotion of events at the chromosomes after completion of processes elsewhere in the cell, in particular, resolution of recombination intermediates and relaxation of sister chromatid cohesion in the chromosome arms at meiosis I.

The persistence of phosphorylated Rec8p after meiosis I may be required for cohesion of the centromeres, as proposed for the phosphoprotein Cor1/SCP3 of rats. Cor1 relocates from the dissolving lateral elements to the centromeric regions of chromosomes and persists there until anaphase II (20, 53). A role in centromere cohesion before anaphase II has been established for the Mei-S332 protein of *Drosophila* (36).

Evaluation of the hypothesis on primary Rec8p functions. First, we consider early Rec8p functions. An alternative to the model presented above may propose binding of Rec8p to specific sites on replicated chromosomes and subsequent formation of homologous contacts, leading to linear-element formation and recombination initiation. If this equivalent model applies, it may also be proposed that Rec8p binds to sites of sister chromatid cohesion formed during premeiotic DNA replication by the same mechanism as in mitotic DNA replication and thus involving mitotic cohesins. Scc1p/Mcd1p, the *S. cerevisiae* homolog of Rad21p, was reported to bind to chromosomes during mitotic S phase (28, 49).

A number of proteins involved in recombination were shown

to form foci on the chromosomes during meiotic prophase (see, e.g., references 10 and 72). It remains to be demonstrated whether Rec8p foci colocalize with recombination enzymes loading onto the initiation sites of recombination. We propose loading of Rec8p onto emerging recombination initiation complexes as a parsimonious way to ensure that linear-element formation occurs at the sites where stabilization of recombination intermediates is required. Ultimately, sister chromatid cohesion in the chromosome arms is required after crossover formation and recombination complex (nodule) dissolution, and it must then be maintained up to anaphase I. However, sister chromatid cohesion may also have a function in early prophase of fission yeast meiosis. Its presence in wild-type early prophase and its absence in *rec8* mutant prophase have been demonstrated (54, 70).

With Rec8p antisera, the protein was localized to about 100 foci in prophase nuclei (Fig. 3). The average number of crossovers per fission yeast meiosis is 45 (56). Thus, sufficient Rec8p foci form during prophase to account also for conversion events not associated with crossing over. A roughly 1:2 relation between crossover and conversion events was proposed early in the analysis of intragenic recombination of *S. cerevisiae* (for a review, see reference 61) and is consistent with the existing data on *S. pombe* (88). Smith and Roeder (77) proposed that Red1 protein nucleates the formation of axial elements in *S. cerevisiae* and plays a role in meiotic sister chromatid cohesion. Red1 also interacts with a protein phosphatase (83). The respective roles of Red1 and the *S. cerevisiae* Rec8p homolog (see below) remain to be investigated.

As reviewed by Kleckner (37), the lateral elements may stabilize the initial homologous contacts by firmly organizing the two sister chromatids attached to them. DNA regions close to the sites of attachment of chromatin loops to the lateral elements may then be protected for maintenance of their interactions with one of the homologous chromatids throughout prophase, until the recombination intermediates are resolved. Genetic and cytological data collected on *rec8* mutants clearly demonstrated the involvement of Rec8p in sister chromatid cohesion and consequently in correct chromosome segregation in meiosis I (54). Rec8p may also be involved in late sister chromatid cohesion that is required for maintenance of chiasmata until transition from metaphase to anaphase I (for a review, see reference 52). Late sister chromatid cohesion must be independent of linear elements, since degradation of linear elements clearly occurs before meiosis I (2). Concurrently, nuclei approaching meiosis I also stop nuclear movement (13, 81).

Rec8p is phosphorylated during meiotic prophase. Its mitotic homolog, Rad21p, is multiply phosphorylated on serine and threonine residues, and its phosphorylation status varied through the cell cycle (9). The transition from unphosphorylated to phosphorylated Rec8p occurs early in prophase (Fig. 4), perhaps in connection with linear-element polymerization. Additional (de)phosphorylation steps may occur at the transitions to completion of recombination and linear-element degradation. The presence of Rec8p even after meiosis I suggests a further role for the protein that may be similar to the one proposed for the rat SCP3/Cor1 lateral-element component. SCP3/Cor1 is a phosphoprotein whose phosphorylation state changes during pachytene (43). Relocation of Rec8p to the centromeres of chromosomes, as demonstrated for Cor1 in rat meiosis (20, 53), is consistent with our results in Fig. 4. Recently, Watanabe and Nurse (87) obtained evidence for binding of Rec8p to the centromeric regions of chromosomes before meiosis I and persistence of this binding up to meiosis II. Their work emphasizes the function of Rec8p at the centromeric regions of the chromosomes, but they also observed localization of Rec8p throughout the nucleus during meiotic prophase.

Region specificity of recombination reduction in *rec8* mutants. De Veaux and Smith (18) showed that *rec8*, *rec10*, and *rec11* point mutations reduce the recombination frequency more than 100-fold in the central part of chromosome III but not more than 10-fold in chromosomes I and II. They proposed that the respective wild-type proteins activate recombination in some chromosome regions but not in others. Our additional data showing strong reduction also in the central regions of chromosomes II and I (Fig. 2), as well as similar results obtained independently (42), do not exclude this hypothesis. However, the results are at odds with our hypothesis proposing that Rec8p binds to all early homologous contacts that will be resolved as recombination events. We suggest an alternative explanation, assuming that several factors contribute to the high level of meiotic recombination and that they are of variable importance in different regions of the genome.

Meiotic recombination frequencies are 2 to 3 orders of magnitude above the mitotic recombination frequencies in fission yeast (50), as in other organisms (3). Disruption of spindle pole integrity and telomere clustering leads to about a fivefold reduction of recombination frequencies (15, 59, 73). A similar result was obtained when nuclear movement was abolished (31). Thus, formation and maintenance of the bouquet and nuclear movement are two factors contributing to high levels of meiotic recombination. However, this contribution is minor compared to other factors. We suggest that the vigorous nuclear movement may also act negatively, leading to disruption of homologous pairing before recombination intermediates are resolved. This would reduce recombination frequencies most strongly in regions that are under greater mechanical stress.

Our model for primary Rec8p function proposes that linear elements contribute to the stabilization of recombination intermediates. The small reduction of recombination in the vicinity of the telomeres (Fig. 2) may then result from maintenance of pairing due to telomere clustering and little disruption by chromosome movement. In contrast, in the regions far from the telomeres, the loss of stabilization by linear elements may frequently lead to disruption of recombination intermediates due to mechanical stress. While recombination reduction is larger than 2 orders of magnitude in the centromeric regions of chromosomes II and III, it is less pronounced in the centromeric region of chromosome I. Interestingly, both arms of chromosome III and one arm of chromosome II are short, while both arms of chromosome I are longer. Long arms may experience less mechanical stress than short arms.

An alternative explanation for the strong region-specific reduction of recombination frequency in *rec8* mutant strains may be the involvement of another protein, fulfilling similar functions to Rec8p only at the ends of chromosomes. However, the slight reduction of recombination frequencies at the telomere regions in *rec8* mutant strains indicates that Rec8p also has a function at the ends of chromosomes.

The *rad21/rec8* gene family and cloning of a human *rec8* homolog. On the basis of amino acid similarity to Rec8p, we identified a human homolog of fission yeast *rec8*. *hrec8* was homologous both to *rec8* and *rad21* of fission yeast and to the previously described human *rad21* homolog (48) (see above). The degree of homology is well within the range previously found for orthologous yeast-mammalian DNA repair and metabolism genes (23).

We also identified a *rec8* homolog, *rec8*^{sc}, within the published budding-yeast genome. Interestingly, disruption of the *S. cerevisiae rec8*^{sc} gene resulted in no detectable phenotype

under vegetative growth (spore germination, growth on different media, and resistance to UV radiation and methyl methanesulfonate). However, homozygous deletion of *rec8*^{sc} resulted in loss of sporulation (4).

Given the sequence homology between *rad21* and *rec8* family members, which genes are true homologs in the different species? For a number of reasons, it is likely that the designation we use here is correct. First, in the regions of conserved sequence, there are key residues conserved between the *rec8* family members which are not conserved in the *rad21* group, and vice versa. For example, with reference to human Hrec8p (Fig. 5A and B), the residues at position 17 are T or V in *rec8* genes but mostly K in *rad21* genes; at position 25, *rec8* genes have all G residues whereas the others have different amino acids; at position 94, H and S are present in *rec8* genes and mostly E is present in *rad21* genes. Likewise, T23 and S26 are conserved between Rec8p and Hrec8p, but the *rad21* genes carry a chemically different amino acid. Second, deletion of the *rad21* and *SCC1/MCD1* genes in fission and budding yeasts revealed that they are essential for mitotic growth. Hypomorphic alleles show similar phenotypes in mitotic cells (8, 28, 49). In contrast, deletion of the *rec8* genes in both yeast species resulted in nonvital phenotypes restricted to meiosis and sporulation (reference 4 and our results). Third, Hrec8p may partially complement the low spore viability of a fission yeast *rec8* deletion strain but fails to complement recombination deficiency.

We did not find a clear separation of *rad21* from *rec8* classes on phylogenetic analysis of the *rad21/rec8* gene family. For some duplicated genes involved in DNA metabolism, e.g., *RAD23* and *RAD6* of *S. cerevisiae* (40, 84), gene duplication apparently occurred during the one billion or more years of evolution from yeasts to mammals (38). For the *rad21/rec8* family, however, it appears that gene duplication occurred earlier, before differentiation of yeasts and other eukaryotes, probably with the evolution of meiosis itself. Further, as evidenced by the lengths of the lines in Fig. 5C, the *rec8* genes appear to have evolved at a higher rate than the corresponding *rad21* genes in the respective species. The common root between the two *C. elegans* proteins and hHR21^{sp} (Fig. 5C) also suggests that these sequences are derived from the same ancestral gene. Since *rad21* and *rec8* genes exist in budding yeast, fission yeast, *Drosophila* (85), and mammals, they are likely to be present in intermediate species as well. Thus, a *rec8* homolog may eventually be identified in *C. elegans*.

hrec8 was mapped by FISH to chromosome 14q11.2-12 (Fig. 6A). This locus does not correspond to any known human disease. Previously, sequence-tagged sites corresponding to *hrec8* were mapped to chromosomes 1, 5, 14, and X (Genethon). The mapping to X may represent cross-hybridization to the *hHR21*^{sp} intronless pseudogene located on the X chromosome (GenBank accession no. HSU85A3). Apparently this pseudogene originated recently in evolution, given the high degree of identity to *hHR21*^{sp} (data not shown).

As with the mouse *rad21* homolog, *mHR21*^{sp}, mRNA formation from *mrec8* was high in testis tissue and detectable above baseline in thymus tissue. Recombination occurs in both these tissues. However, unlike *mHR21*^{sp}, *mrec8* was highly expressed in postmeiotic testis tissue (Fig. 6B). The transcript seen in postmeiotic cells did not represent cross-hybridization of *mrec8* with the *mHR21*^{sp} gene, since *mHR21*^{sp} mRNAs differ in size and since nucleotide sequence similarity between these genes is poor in the region of the probe used for hybridization (data not shown). The significance of *mrec8* expression in postmeiotic testis cells is unknown. However, in view of the role for a *rad21/rec8* family member in chromatin condensation (28), *mrec8* may be involved in the marked chromatin repackaging accompanying spermiogenesis.

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The first two authors contributed equally to this work.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
2. **Bähler, J., T. Wyler, J. Loidl, and J. Kohli.** 1993. Unusual nuclear structures in meiotic prophase of fission yeast: a cytological analysis. J. Cell Biol. **121**:241–256.
3. **Baker, B. S., A. T. C. Carpenter, M. S. Esposito, R. E. Esposito, and L. Sandler.** 1976. The genetic control of meiosis. Annu. Rev. Genet. **10**:53–134.
4. **Bashkirov, V., and W.-D. Heyer.** Personal communication.
5. **Basi, G., E. Schmid, and K. Maundrell.** 1993. TATA box mutations in the *Schizosaccharomyces pombe nmt1* promoter affect transcription efficiency but not the transcription start point or thiamine repressibility. Gene **123**:131–136.
6. **Beach, D., L. Rodgers, and J. Gould.** 1985. *RAN1*$^{+}$ controls the transition from mitotic division to meiosis in fission yeast. Curr. Genet. **10**:297–311.
7. **Bickel, S. E., D. W. Wyman, and T. L. Orr-Weaver.** 1997. Mutational analysis of the *Drosophila* sister-chromatid cohesion protein ORD and its role in the maintenance of centromeric cohesion. Genetics **146**:1319–1331.
8. **Birkenbihl, R. P., and S. Subramani.** 1992. Cloning and characterization of *rad21* an essential gene of *Schizosaccharomyces pombe* involved in DNA double-strand-break repair. Nucleic Acids Res. **20**:6605–6611.
9. **Birkenbihl, R. P., and S. Subramani.** 1995. The *rad21* gene product of *Schizosaccharomyces pombe* is a nuclear, cell-cycle regulated phosphoprotein. J. Biol. Chem. **270**:7703–7711.
10. **Bishop, D. K.** 1994. RecA homologs Rad51 and Dmc1 interact to form discrete nuclear complexes prior to meiotic chromosome synapsis. Cell **79**: 1081–1092.
11. **Bresch, C., G. Müller, and R. Egel.** 1968. Genes involved in meiosis and sporulation of a yeast. Mol. Gen. Genet. **102**:301–306.
12. **Carpenter, A. T. C.** 1979. Recombination nodules and synaptonemal complex in recombination-defective females of *Drosophila melanogaster*. Chromosoma **75**:259–292.
13. **Chikashige, Y., D. Q. Ding, H. Funabiki, T. Haraguchi, S. Mashiko, M. Yanagida, and Y. Hiraoka.** 1994. Telomere-led premeiotic chromosome movement in fission yeast. Science **264**:270–273.
14. **Cohen-Fix, O., J. M. Peters, M. W. Kirschner, and D. Koshland.** 1996. Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. Genes Dev. **10**: 3081–3093.
15. **Cooper, J. P., Y. Watanabe, and P. Nurse.** 1998. Fission yeast Taz1 protein is required for meiotic telomere clustering and recombination. Nature **392**: 828–831.
16. **Cottarel, G., D. Beach, and U. Deuschle.** 1993. Two new multipurpose multicopy *Schizosaccharomyces pombe* shuttle vectors, pSP1 and pSP2. Curr. Genet. **23**:547–548.
17. **De Veaux, L. C., N. A. Hoagland, and G. R. Smith.** 1992. Seventeen complementation groups of mutations decreasing meiotic recombination in *Schizosaccharomyces pombe*. Genetics **130**:251–261.
18. **De Veaux, L. C., and G. R. Smith.** 1994. Region-specific activators of meiotic recombination in *Schizosaccharomyces pombe*. Genes Dev. **8**:203–210.
19. **Dirick, L., L. Goetsch, G. Ammerer, and B. Byers.** 1998. Regulation of meiotic S phase by Ime2 and a Clb5,6-associated kinase in *Saccharomyces cerevisiae*. Science **281**:1854–1857.
20. **Dobson, M. J., R. E. Pearlman, A. Karaiskakis, B. Spyropoulos, and P. B. Moens.** 1994. Synaptonemal complex proteins: occurrence, epitope mapping and chromosome disjunction. J. Cell Sci. **107**:2749–2760.

21. **Egel, R., and M. Egel-Mitani.** 1974. Premeiotic DNA synthesis in fission yeast. Exp. Cell Res. **88**:127–134.
22. **Felsenstein, J.** 1993. PHYLIP, version 3.5c. Department of Genetics, University of Washington, Seattle.
23. **Friedberg, E. C., A. J. Bardwell, L. Bardwell, W. J. Feaver, R. D. Kornberg, J. Q. Svejstrup, A. E. Tomkinson, and Z. Wang.** 1995. Nucleotide excision repair in the yeast *Saccharomyces cerevisiae*: its relationship to specialized mitotic recombination and RNA polymerase II basal transcription. Philos. Trans. R. Soc. London Ser. B **347**:63–68.
24. **Funabiki, H., H. Yamano, K. Kumada, K. Nagao, T. Hunt, and M. Yanagida.** 1996. Cut2 proteolysis required for sister-chromatid separation in fission yeast. Nature **381**:438–441.
25. **Grimm, C., J. Kohli, J. Murray, and K. Maundrell.** 1988. Genetic engineering of *Schizosaccharomyces pombe*. A system for gene disruption and replacement using the *ura4* gene as selectable marker. Mol. Gen. Genet. **215**:81–86.
26. **Grimm, C., P. Schär, P. Munz, and J. Kohli.** 1991. The strong *adh* promoter stimulates mitotic and meiotic recombination at the *ade6* gene of *Schizosaccharomyces pombe*. Mol. Cell. Biol. **11**:289–298.
27. **Grootegoed, J. A., R. Janson, and H. J. van der Molen.** 1986. Effect of glucose on ATP dephosphorylation in rat spermatids. J. Reprod. Fertil. **77**:99–107.
28. **Guacci, V., D. Koshland, and A. Strunnikov.** 1997. A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD1 in *S. cerevisiae*. Cell **91**:47–57.
29. **Gutz, H., H. Heslot, U. Leupold, and N. Loprieno.** 1974. *Schizosaccharomyces pombe*, p. 395–446. *In* R. C. King (ed.), Handbook of genetics, vol. 1. Plenum Press, New York, N.Y.
30. **Hari, K. L., A. Santerre, J. J. Sekelsky, K. S. McKim, J. B. Boyd, and R. S. Hawley.** 1995. The *mei-41* gene of *D. melanogaster* is a structural and functional homolog of the human ataxia telangiectasia gene. Cell **82**:815–821.
31. **Hiraoka, Y.** Personal communication.
32. **Huysmans, E., E. Dams, A. Vandenberghe, and R. De Wachter.** 1983. The nucleotide sequence of the 5S rRNAs of four mushrooms and their use in studying the phylogenetic position of basidiomycetes among the eukaryotes. Nucleic Acids Res **11**:2871–2880.
33. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact cells treated with alkali cations. J. Bacteriol. **153**:487–493.
34. **Kato, R., and H. Ogawa.** 1992. An essential gene, *ESR1*, is required for mitotic cell growth, DNA repair and meiotic recombination in *Saccharomyces cerevisiae*. Nucleic Acids Res. **22**:3104–3112.
35. **Kerrebrock, A. W., W. Y. Miyazaki, D. Birnby, and T. L. Orr-Weaver.** 1992. The *Drosophila mei-S332* gene promotes sister-chromatid cohesion in meiosis following kinetochore differentiation. Genetics **130**:827–841.
36. **Kerrebrock, A. W., D. P. Moore, J. S. Wu, and T. L. Orr-Weaver.** 1995. Mei-S332, a *Drosophila* protein required for sister-chromatid cohesion, can localize to meiotic centromere regions. Cell **83**:247–256.
37. **Kleckner, N.** 1996. Meiosis: how could it work? Proc. Natl. Acad. Sci. USA **93**:8167–8174.
38. **Knoll, A. H.** 1992. The early evolution of eukaryotes: a geological perspective. Science **256**:622–627.
39. **Kohli, J.** 1994. Telomeres lead chromosome movement. Curr. Biol. **4**:724–727.
40. **Koken, M. H., P. Reynolds, I. Jaspers-Dekker, L. Prakash, S. Prakash, D. Bootsma, and J. H. Hoeijmakers.** 1991. Structural and functional conservation of two human homologs of the yeast DNA repair gene RAD6. Proc. Natl. Acad. Sci. USA **88**:8865–8869.
41. **Kozak, M.** 1981. Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acids Res. **9**:5233–5252.
42. **Krawchuk, M. D., and W. P. Wahls.** Personal communication.
43. **Lammers, J. H. M., M. van Alderen, A. H. F. M. Peters, A. A. M. van Pelt, I. C. Gaemmers, D. G. de Rooij, P. de Boer, H. H. Offenberg, A. J. J. Dietrich, and C. Heyting.** 1995. A change in the phosphorylation pattern of the 30000 to 33000 M_r synaptonemal complex proteins of the rat between early and mid-pachytene. Chromosoma **104**:154–163.
44. **Leem, S.-H., and H. Ogawa.** 1992. The *MER4* gene encodes a novel protein kinase homologue required for meiotic recombination in *Saccharomyces cerevisiae*. Nucleic Acids Res. **20**:449–457.
45. **Lin, Y., D. Evans, and G. R. Smith.** Personal communication.
46. **Lin, Y., K. L. Larson, R. Dorer, and G. R. Smith.** 1992. Meiotically induced *rec7* and *rec8* genes of *Schizosaccharomyces pombe*. Genetics **132**:75–85.
47. **Lydall, D., Y. Nikolsky, D. K. Bishop, and T. Weinert.** 1996. A meiotic recombination checkpoint controlled by mitotic checkpoint genes. Nature **383**:840–843.
48. **McKay, M. J., C. Troelstra, P. van der Spek, R. Kanaar, B. Smit, A. Hagemeijer, D. Bootsma, and J. H. Hoeijmakers.** 1996. Sequence conservation of the *rad21 Schizosaccharomyces pombe* DNA double-strand break repair gene in human and mouse. Genomics **36**:305–315.
49. **Michaelis, C., R. Ciosk, and K. Nasmyth.** 1997. Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell **91**:35–45.
50. **Minet, M., A.-M. Grossenbacher-Grunder, and P. Thuriaux.** 1980. The origin of a centromere effect on mitotic recombination. Curr. Genet. **2**:53–60.
51. **Miyazaki, W. Y., and T. L. Orr-Weaver.** 1992. Sister-chromatid misbehavior in *Drosophila ord* mutants. Genetics **132**:1047–1061.
52. **Miyazaki, W. Y., and T. L. Orr-Weaver.** 1994. Sister-chromatid cohesion in mitosis and meiosis. Annu. Rev. Genet. **28**:167–187.
53. **Moens, P. B., and B. Spyropoulos.** 1995. Immunocytology of chiasmata and chromosomal disjunction at mouse meiosis. Chromosoma **104**:175–182.
54. **Molnar, M., J. Bähler, M. Sipiczki, and J. Kohli.** 1995. The *rec8* gene of *Schizosaccharomyces pombe* is involved in linear element formation, chromosome pairing and sister-chromatid cohesion during meiosis. Genetics **141**:61–73.
55. **Moreau, P. J. F., D. Zickler, and G. Leblon.** 1985. One class of mutants with disturbed centromere cleavage and chromosome pairing in *Sordaria macrospora*. Mol. Gen. Genet. **198**:189–197.
56. **Munz, P.** 1994. An analysis of interference in the fission yeast *Schizosaccharomyces pombe*. Genetics **137**:701–707.
57. **Munz, P., and U. Leupold.** 1979. Gene conversion in nonsense suppressors of *Schizosaccharomyces pombe*. I. The influence of the genetic background and of three mutant genes (*rad2*, *mut1* and *mut2*) on the frequency of postmeiotic segregation. Mol. Gen. Genet. **170**:145–148.
58. **Nadin-Davis, S. A., and A. Nasim.** 1990. *Schizosaccharomyces pombe ras1* and *byr1* are functionally related genes of the *ste* family that affect starvation-induced transcription of mating-type genes. Mol. Cell. Biol. **10**:549–560.
59. **Nimmo, E. R., A. L. Pidoux, P. E. Perry, and R. C. Allshire.** 1998. Defective meiosis in telomere-silencing mutants of *Schizosaccharomyces pombe*. Nature **392**:825–828.
60. **Pawson, T., and J. Schlessinger.** 1993. SH2 and SH3 domains. Curr. Biol. **3**:434–442.
61. **Petes, T. D., R. E. Malone, and L. S. Symington.** 1991. Recombination in yeast, p. 407–521. *In* J. R. Broach, J. R. Pringle, and E. E. Jones (ed.), The molecular and cellular biology of the yeast *Saccharomyces cerevisiae*: genome dynamics, protein synthesis and energetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
62. **Pinkel, D., T. Straume, and J. W. Gray.** 1986. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc. Natl. Acad. Sci. USA **83**:2934–2938.
63. **Ponticelli, A. S., and G. R. Smith.** 1989. Meiotic recombination-deficient mutants of *Schizosaccharomyces pombe*. Genetics **123**:45–54.
64. **Prabhala, G., G. H. Rosenberg, and N. F. Käufer.** 1992. Architectural features of pre-mRNA introns in the fission yeast *Schizosaccharomyces pombe*. Yeast **8**:171–182.
65. **Pringle, J. R., A. E. M. Adams, D. G. Drubin, and B. K. Haarer.** 1991. Immunofluorescence methods for yeast. Methods Enzymol. **194**:565–602.
66. **Rechsteiner, M., and S. W. Rogers.** 1996. PEST sequences and regulation by proteolysis. Trends Biochem. Sci. **21**:267–271.
67. **Rockmill, B., and G. S. Roeder.** 1991. A meiosis-specific protein kinase homolog required for chromosome synapsis and recombination. Genes Dev. **5**:2392–2404.
68. **Roeder, G. S.** 1997. Meiotic chromosomes: it takes two to tango. Genes Dev. **11**:2600–2621.
69. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
70. **Scherthan, H., J. Bähler, and J. Kohli.** 1994. Dynamics of chromosome organization and pairing during meiotic prophase in fission yeast. J. Cell Biol. **127**:273–285.
71. **Schweingruber, M. E., and E. Edenharter.** 1990. Thiamin regulates agglutination and zygote formation in *Schizosaccharomyces pombe*. Curr. Genet. **17**:191–194.
72. **Scully, R., J. Chen, A. Plug, Y. Xiao, D. Weaver, J. Feunteun, T. Asley, and D. M. Livingston.** 1997. Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell **88**:265–275.
73. **Shimanuki, M., F. Miki, D.-Q. Ding, Y. Chikashige, Y. Hiraoka, T. Horio, and O. Niwa.** 1997. A novel fission yeast gene, *kms1*$^+$, is required for the formation of meiotic prophase-specific nuclear architecture. Mol. Gen. Genet. **254**:238–249.
74. **Shimoda, C., A. Hirata, M. Kishida, T. Hashida, and K. Tanaka.** 1985. Characterization of meiosis-deficient mutants by electron microscopy and mapping of four essential genes in the fission yeast *Schizosaccharomyces pombe*. Mol. Gen. Genet. **200**:252–257.
75. **Shuster, E. O., and B. Byers.** 1989. Pachytene arrest and other meiotic effects of the start mutations in *Saccharomyces cerevisiae*. Genetics **123**:29–43.
76. **Sipiczki, M.** 1989. Taxonomy and phylogenesis, p. 431–452. *In* A. Nasim, P. Young and B. F. Johnson (ed.), Molecular biology of the fission yeast. Academic Press, Inc., New York, N.Y.
77. **Smith, A. V., and G. S. Roeder.** 1997. The yeast Red1 protein localizes to the cores of meiotic chromosomes. J. Cell Biol. **136**:957–967.
78. **Smith, D. B., and K. S. Johnson.** 1988. Single step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67**:31–40.
79. **Soares, M. B., M. F. Bonaldo, P. Jelene, L. Su, L. Lawton, and A. Efstra-**

tiadis. 1994. Construction and characterization of a normalized cDNA library. Proc. Natl. Acad. Sci. USA **91**:9228–9232.
80. **Strauss, A.** 1979. Isolation and genetic classification of aromatic amino acid auxotrophic mutants in *Schizosaccharomyces pombe*. J. Gen. Microbiol. **113**: 173–176.
81. **Svoboda, A., J. Bähler, and J. Kohli.** 1995. Microtubule-driven nuclear movement and linear elements as meiosis-specific characteristics of the fission yeasts *Schizosaccharomyces versatilis* and *Schizosaccharomyces pombe*. Chromosoma **104**:203–214.
82. **The *C. elegans* Sequencing Consortium.** 1998. Genome sequence of the nematode *C. elegans*: a platform for investigating biology. Science **282**:2012–2018.
83. **Tu, J., W. Song, and M. Carlson.** 1996. Protein phosphatase type I interacts with proteins required for meiosis and other cellular processes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16**:4199–4206.
84. **van der Spek, P. J., C. E. Visser, F. Hanaoka, B. Smit, A. Hagemeijer, D. Bootsma, and J. H. Hoeijmakers.** 1996. Cloning, comparative mapping, and RNA expression of the mouse homologues of the *Saccharomyces cerevisiae* nucleotide excision repair gene *RAD23*. Genomics **31**:20–27.
85. **Warren, W.** Personal communication.
86. **Watanabe, Y., Y. Iino, K. Furuhata, C. Shimoda, and M. Yamamoto.** 1988. The *S. pombe mei2* gene encoding a crucial molecule for commitment to meiosis is under the regulation of cAMP. EMBO J. **7**:761–767.
87. **Watanabe, Y., and P. Nurse.** Personal communication.
88. **Zahn-Zabal, M., E. Lehmann, and J. Kohli.** 1995. Hot spots of recombination in fission yeast: inactivation of the *M26* hot spot by deletion of the *ade6* promoter and the novel hotspot *ura4-aim*. Genetics **140**:469–478.